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The Signaling Pathway of Oxysterol Induced Apoptosis
in Chinese Hamster Ovary (CHO)-K1 Cells

A dissertation
presented to
the faculty of the Department of
Biochemistry and Molecular Biology
James H. Quillen College of Medicine
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy in Biomedical Sciences

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August 2002

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Cytosolic Phospholipase A2, Calcium, Prostaglandin, PPAR Gamma, Signaling Pathway

ABSTRACT

The Signaling Pathway of Oxysterol Induced Apoptosis in Chinese Hamster Ovary (CHO)-K1 Cells

by

Lin Yang

Apoptosis, a form of genetically programmed cell death, plays a key role in regulation of cellularity of the arterial wall. During atherogenesis, improper apoptosis may cause abnormalities of arterial morphogenesis, wall structural stability, and metabolisms. It has been well established that vascular cells undergo apoptosis after uptake of oxidized low-density lipoprotein (oxLDL). Thus, an analysis of the signaling pathway of apoptotic induction by oxLDL is of value in understanding the development of atherosclerotic plaque. In order to elucidate the signaling pathway of apoptosis induced by oxLDL, we have used Chinese Hamster Ovary (CHO)-K1 cells treated with a potent oxysterol, 25-hydroxycholesterol (25-OHC). In the present study, we find that oxLDL can induce apoptosis in any cell types if cells present the specific receptors on their surface to take up oxLDL and that apoptosis-inducing activity is associated with oxysterol components in oxLDL. Oxysterol-induced apoptosis does not involve regulation of sterol regulatory element-binding protein proteolysis pathway. 25-OHC stimulates calcium uptake by CHO-K1 cells within 2 min after addition. Treatment of CHO-K1 cells with the calcium channel blocker nifedipine prevents 25-OHC induction of apoptosis. One possible signal transduction pathway initiated by calcium ion fluxes is the activation of cytosolic phospholipase A₂ (cPLA₂). We demonstrate that activation of cPLA₂ does occur in CHO-K1 treated with 25-OHC. Activation is evidenced by 25-OHC-induced relocalization of cPLA₂ to the nuclear envelope and arachidonic acid (AA) release. Loss of cPLA₂ activity by treatment with a cPLA₂ inhibitor results in an attenuation of AA release as well as of the apoptotic response to 25-OHC in CHO-K1 cells. cPLA₂-mediated liberation of AA leads to the formation of a cyclooxygenase product, probably a prostaglandin, which activates the transcription factor PPAR γ and induces apoptosis. We also examined the execution phase of the apoptotic pathway in CHO-K1 cell death induced by 25-OHC. Oxysterol-induced apoptosis is accompanied by caspase activation and is preceded by mitochondrial cytochrome C release. Furthermore, treatment with a cPLA₂ inhibitor results in an inhibition of caspase-3 activation in CHO-K1 cells. These data provide strong evidence indicating that 25-OHC induces caspase-3-mediated apoptosis via an activation of calcium-dependent cPLA₂.

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LIST OF ABBREVIATIONS

AA	Arachidonic Acid
AACOCF ₃	Arachidonyltrifluoromethyl ketone
ACAT	Acetyltransferase
BEL	Bromoenol lactone
BSA	Bovine Serum Albumin
CHO	Chinese Hamster Ovary
COX	Prostaglandin G/H synthase
cPLA ₂	Ca ²⁺ -dependent cytosolic PLA ₂
CTLs	Cytotoxic T lymphocytes
15d-PGJ ₂	15-Deoxyl- $\Delta^{12, 14}$ -prostaglandin J ₂
(E)GFP	(Enhanced) Green Fluorescent Protein
ER	Endoplasmic Reticulum
EtOH	Ethanol
ETYA	5,8,11,14-Eicosatetraenoic acid
F12FC5	Ham's F-12 medium containing 5 % fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin
HDL	High-Density Lipoprotein
HMG-CoA	3-Hydroxy-3-Methylglutaryl Coenzyme A
HPLC	High Performance Liquid Chromatography
iPLA ₂	Ca ²⁺ -independent PLA ₂
LDL	Low-Density Lipoprotein
MAP kinases	Mitogen-Activated Protein kinases
NF κ B	Nuclear Factor κ B
25-OHC	25-Hydroxycholesterol
OxLDL	Oxidized Low-Density Lipoprotein
OX ^R	25-Hydroxycholesterol-Resistant Mutant Cell Line
PARP	Poly (ADP-ribose) Polymerase
PBS	Phosphate-Buffered Saline
PPAR	Peroxisome proliferator-activated receptors
PPRE	Peroxisome Proliferator Response Element
PR-HPLC	Reverse phase (RP) high-performance liquid chromatography
SMC	Smooth Muscle Cell
sPLA ₂	Ca ²⁺ -dependent secretory PLA ₂
SREBP	Sterol Regulatory Element Binding Proteins
TBS-T	Tris-buffered saline containing 0.1% Tween 20
TE buffer	Tris-EDTA buffer
TNF	Tumor Necrosis Factor
TUNEL	Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End Labeling
VLDL	Very Low-Density Lipoprotein

CHAPTER 1

INTRODUCTION

Apoptosis and Oxysterols

Apoptosis

For every cell, there is a time to live and a time to die. Cell death can occur by either of two distinct mechanisms, necrosis or apoptosis. Necrosis ("accidental" cell death) is the pathological process, which occurs when cells are exposed to a serious physical or chemical insult. Apoptosis ("normal" or "programmed" cell death) is the normal physiological process that offsets cell proliferation. The purpose of this process is to kill unwanted host cells in situations like development, homeostasis, defense, and aging (Bredesen 2000). It (Steller 1995) allows cells to self-destruct when stimulated by the appropriate trigger. As a simple analogy, necrosis can be thought of as murder, while apoptosis can be thought of as suicide.

There are many observable morphological and biochemical differences between necrosis and apoptosis (Vermes and Haanan 1994). Necrosis begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, *in vivo*, necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response (Van Furth and Van Zwet 1988). Cells undergoing apoptosis show characteristic morphological and biochemical features (Cohen 1993). These features include membrane blebbing, chromatin aggregation, nuclear and cytoplasmic condensation, DNA fragmentation,

partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material. *In vivo*, these apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells (Savill *et al.* 1989). Due to this efficient mechanism for the removal of apoptotic cells *in vivo* no inflammatory response is elicited.

There is intense current interest in studies of apoptosis, with a recent exponential expansion in PubMed textword citations. Apoptosis research has been spurred by the observation that normal metazoan development and health require the precise regulation of cell death (Harmon *et al.* 1984; Lynch *et al.* 1986). Historically, embryologists in the early 20th century were familiar with the process of apoptosis. They observed that as an embryo develops, many of its cells are sacrificed to create the final form of the organism. Not until 1972, however, did researchers Kerr *et al.* recognize the broader significance of this mechanism. They coined the term apoptosis from the Greek word meaning "falling off," as leaves do in autumn, to describe this natural, timely death of cells.

Apoptosis is a genetically programmed event that can be set in motion by a variety of internal or external stimuli. It is initiated for two different reasons: 1) Programmed cell death is needed for proper development as is mitosis. It usually occurs when a cell is no longer needed within the body. For example, the formation of the fingers and toes of the fetus requires the removal, by apoptosis, of the tissue between them; The sloughing off of the inner lining of the uterus (the endometrium) at the start of menstruation occurs by apoptosis; and the formation of the proper connections (synapses) between neurons in the brain requires that surplus cells be eliminated by apoptosis. 2) Programmed cell death is needed to destroy cells that represent a threat to the health of the organism. For example, when cells are infected

with viruses, cytotoxic T lymphocyte (CTLs) kill these virus-infected cells by inducing apoptosis. As cell-mediated immune responses wane, the effector cells must be removed to prevent them from attacking body constituents. CTLs induce apoptosis in each other and even in themselves (Berke 1991; Krahenbuhl and Tschopp 1991).

Apoptosis plays a critical role in important biological processes (Steller 1995). It is required to maintain the balance between cell proliferation and cell death in embryonic development as well as in the daily maintenance of a mature organism. The aberrant inhibition or initiation of apoptosis contributes to many disease processes. Various phenomena can disturb the regulation of the cell death pathway, causing too many or too few cells to die. Impaired apoptosis may be a significant factor in the etiology of such diseases as cancer, autoimmune disorders, and viral infections (Meygaard *et al.* 1992). If, for example, a mutation occurs in a gene that induces apoptosis, such as the tumor-suppressor gene p53, the cell that harbors the gene may fail to respond to the cue to die. As a result the cell may proliferate uncontrollably and form a cancerous tumor (Clarke *et al.* 1993; Lowe *et al.* 1993). In other cases a virus may interfere with the regulation of apoptosis, inducing healthy cells to die. This mechanism is believed to play a role in AIDS, the disease in which infection with the HIV virus results in the destruction of healthy white blood cells called T lymphocytes (Dockrell 2001; Frost and Michie 1996). A major goal of apoptosis research is to identify its molecular components and mechanisms of regulation. This information may lead to therapeutic agents that can modulate this process in the treatment of degenerative diseases.

Apoptosis in Atherosclerosis

The general term for hardening of the arteries is atherosclerosis (Montgomery *et al.* 1977). In the healthy artery, the lumen of the normal artery is free of obstruction, permitting the flow of blood, and the layers of the artery (the endothelium, the media and the adventitia) are intact and free of cellular or particulate intrusions. In the atherosclerotic artery, the lumen of the pathological artery is almost completely

occluded and the flow of blood within such an artery is blocked. If a coronary artery is occluded, that may result in a heart attack. Atherosclerosis is the main cause of death for both men and women in American. About half of all people with a Western lifestyle are currently dying of myocardial infarcts or strokes caused by sudden occlusion of arteries narrowed by atherosclerotic plaques (Berliner and Heinecke 1996; Esterbauer *et al.* 1992).

Atherogenesis is a slowly progressive disease (Stary 1989). It is formed by vascular cell hyperplasia or hypertrophy and matrix protein accumulation in the intima (the innermost layer of a blood vessel) and /or media with lipid deposition, resulting in thickening and stiffness of the arterial wall (Steinberg and Witztum 1990; Ross 1993). The atherosclerotic lesion is defined by arterial intima and smooth muscle cell (SMC) proliferation, lipid accumulation, and connective tissue deposition. Depending on their size and composition, lesions are usually divided into fatty streaks, early stages of lesions, and advanced stages of atherosclerosis called plaques. The three major cellular components of human atherosclerotic plaques are vascular smooth muscle cells (SMCs), which dominate the fibrous cap; macrophages, which are the most abundant cell type in the lipid-rich core; and lymphocytes, which have been mainly ascribed to the fibrous cap (Ross 1993).

Apoptosis plays a key role in regulation of cellularity of the arterial wall. During atherogenesis, deregulated apoptosis may cause abnormalities of arterial morphogenesis, wall structural stability, and metabolisms. Recent studies demonstrate the presence of apoptotic cells in atherosclerotic lesions (Bennett *et al.* 1995; Geng and Libby 1995; Han *et al.* 1995; Kockx 1998). Apoptotic cells in atherosclerotic lesions were restricted to the intima of the diseased arteries (Geng and Libby 1995). In these pathological situations apoptosis may not only contribute to alteration of cell number but

may also contribute to the formation of necrotic core and atherosclerotic plaque rupture (Mitchinson *et al.* 1996).

Oxidized Low Density Lipoprotein (oxLDL)-Induced Apoptosis

In the normal course of events, the cholesterol and triglycerides that we ingest during a meal are shuttled through various metabolic pathways in which they can be used in cellular metabolism. Because cholesterol and triglyceride molecules are not soluble in blood they are transported through the blood as complexes in a heterogeneous class of proteins called plasma lipoproteins. Lipoprotein complexes are roughly spherical particles whose cores are neutral lipids that have few or no hydrophilic groups, such as triacylglycerols and cholesterol esters. Lipids that have hydrophilic groups, such as phosphatidylcholine are on the surface of the complexes. The proteins associated with lipoproteins snake their way through the complex with their hydrophobic groups in contact with the lipid phase and their hydrophilic groups in contact with the aqueous phase.

There are 4 major classes of human plasma lipoproteins that are classified by their densities: chylomicrons, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). LDL is one of the most important lipoprotein classes of human serum. The role of LDL is to transport cholesterol to tissues and an elevated level of serum LDL is strongly correlated with atherosclerosis (Rawn 1983). In the late 1970s Michael Brown and Joseph Goldstein (Brown and Goldstein 1974; Brown *et al.* 1986) demonstrated that the receptor for the LDL particle governs the uptake of cholesterol. The complex, between the LDL receptor and lipoprotein, is taken up by the cell through receptor mediated endocytosis. Lysosomes then digest the endocytosed LDL and release cholesterol. Cholesterol can inhibit its own biosynthesis, activate an enzyme that stores cholesterol in cholesterol ester

droplets (acyl CoA cholesterol acyl transferase), and inhibits the synthesis of LDL receptors to assure that the cell will not take up too much cholesterol.

Patients with familial hypercholesterolemia have a mutation in the gene specifying the LDL receptor (Brown and Goldstein 1974). In these patients, LDL degradation through the physiologic pathway is blocked, and each LDL particle survives in the circulation 2-3 times longer than it does in normal subjects. As a result, the lipoprotein accumulates to abnormally high levels in plasma. Eventually the LDL is degraded through an alternate pathway. Brown and Goldstein (Brown *et al.* 1986) have demonstrated that another type of LDL receptor, called the scavenger receptor, is present on macrophages. It turns out, however, that scavenger receptors do not bind normal LDL particles. Instead, as Daniel Steinberg and his colleagues (Steinberg *et al.* 1989) have shown, the scavenger receptor recognizes oxidized LDL particles. Unlike LDL receptors, the expression of the scavenger receptor is not down regulated by high levels of cholesterol within the cell. The resulting accumulation of cholesterol in the macrophages gives these cells the appearance of "foam" cells. The findings that massive accumulations of foam cells are observed in early atherosclerotic lesions support the hypothesis that the unlimited uptake of the oxidatively modified LDL by macrophages is involved in the development of atherosclerosis *in vivo* (Nilsson *et al.* 1992; Noguchi *et al.* 1993; Nilsson 1995). It has, therefore, been proposed that preventing the deleterious "oxidative modification" of LDL should lower the risk of ischemic heart disease (Bowry *et al.* 1995).

OxLDL has also been found to play a role in many early events of atherosclerosis; it induces the expression of adhesion molecules on endothelial cells (Kume *et al.* 1992), the transformation of macrophages and smooth muscle cells to foam cells (Henriksen *et al.* 1981), the production of various proinflammatory cytokines and growth factors by almost all vascular cells (Kume and Gimbrone 1994; Nakano *et*

al. 1994), the proliferation and migration of vascular cells (Quinn *et al.* 1988; Yui *et al.* 1993; Auge *et al.* 1996) and the retardation of endothelial regeneration (Murugesan and Fox 1996), and it increases the procoagulant activity on the vascular cells (Aupeix *et al.* 1996). These changes eventually result in the formation of atheromatous lesions.

OxLDL also affects the later stage of atherosclerosis by its toxicity. It exhibits a dramatic cytotoxic effect on several vascular cell types, including SMCs, macrophages, and endothelial vascular cells (Escargueil-Blanc *et al.* 1997). It has been well established that vascular cells-particularly macrophages (Reid *et al.* 1993; Hardwick *et al.* 1996), and endothelial cells (Dimmeler *et al.* 1997; Escargueil-Blanc *et al.* 1997)-undergo apoptosis after uptake of circulating, oxidized low-density lipoprotein (Mitchinson *et al.* 1996). OxLDL induces both the morphological changes and DNA fragmentation characteristic of apoptosis in cultured smooth muscle cells, macrophages, endothelial cells, and lymphoid cells (Wyllie 1981; Orrenius 1989; Dimmeler *et al.* 1997; Escargueil-Blanc *et al.* 1998; Brown and Jessup 1999). The cytotoxic effect of oxLDL depends on concentration and oxidative modification. High concentrations of oxLDL are pro-apoptotic, whereas low concentrations are mitogenic for vascular SMCs. Regarding the degree of oxidation, Siow *et al.* (1999) provided evidence that moderately oxLDL, with its high lipid hydroperoxide content, seems to be more cytotoxic than mildly or highly oxLDL (Siow *et al.* 1999; Napoli *et al.* 2000). In contrast, protein modifications do not seem to be necessary for LDL-induced cytotoxicity. These results clearly show that the apoptosis-inducing activity is recovered in the neutral lipid fraction of oxLDL.

OxLDL can be generated *in vivo* by at least three classes of mechanisms: 1) autooxidation in the presence of transition metals (Morel *et al.* 1983; Lamb *et al.* 1995;); 2) cell-mediated oxidation (Morel *et al.* 1984; Steinbrecher *et al.* 1984; Hiramatsu *et al.* 1987); and 3) plasma enzyme-mediated oxidation (Parthasarathy *et al.* 1985; Daugherty

et al. 1994; Yla-Herttuala *et al.* 1995). The structural and biological changes in oxLDL may result in lysophospholipids accumulation and lipid peroxidation. Lipid peroxides, particularly oxidized cholesterol derivatives (oxysterols), are known to influence a variety of biological functions *in vitro* (Nishio and Watanabe 1996). Cholesterol oxides have been demonstrated to exist both in oxLDL (Colles *et al.* 1996) and in atherosclerotic plaque (Carpenter *et al.* 1993). Various oxysterols in oxLDL have been shown to induce apoptosis in endothelial cells (Harada-Shiba *et al.* 1998), monocytic cell lines (Aupeix *et al.* 1995), thymocytes (Christ *et al.* 1993), and smooth muscle cells (Ares *et al.* 1997). Therefore, the cytotoxicity of oxLDL can best be accounted for by the formation of cholesterol oxidation products (oxysterol).

Oxysterols

Oxysterols are 27-carbon products of cholesterol oxidation (Brown and Jessup 1999). Through autooxidation or enzymatic reaction, cholesterol and related compounds give rise to a number of sterols containing additional oxygens. Various oxysterols have been detected in appreciable quantities in human tissue and fluids, including human plasma, atherogenic lipoproteins, and atherosclerotic plaque. How these oxysterols originate *in vivo* is not fully known. Some researchers claim that they arise principally from dietary sources while others contend that they are generated by non-enzymatic oxidation *in vivo* (Brown and Jessup 1999).

Oxysterols represent a class of potent regulatory molecules with remarkably diverse, important biological actions. Early research on oxysterols by Kandutsch and Chen (1973, 1974) demonstrated that certain oxygenated derivatives of cholesterol, but not highly purified cholesterol itself, caused an inhibition of sterol biosynthesis and lowering of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity in cultured mammalian cells. These findings stimulated a tremendous amount of research

on oxysterols, including their actions on a wide variety of other processes and the chemical preparation of a large number of natural and synthetic oxygenated sterols. These researches led to the demonstration that some 15-oxygenated sterols were not only extraordinarily potent in suppressing sterol synthesis in cultured cells but also showed significant hypocholesterolemic action upon administration to rodents and nonhuman primates. An increasing amount of evidence indicates that oxysterols represent major natural regulators of sterol synthesis, causing down-regulation of the activity of HMG-CoA reductase (Kandutsch and Chen 1973). Seminal studies by Brown and Goldstein defined the metabolic defect in familial hypercholesterolemia and identified the receptor for LDL (Brown and Goldstein 1975). These discoveries resulted in an explosion of research on LDL, including its roles in the control of sterol biosynthesis and a large variety of other important cellular processes, and studies on the levels of oxysterols in LDL. A considerable amount of research has been focused on the possible involvement of oxysterols, as components of oxidatively modified LDL, in the pathogenesis of atherosclerosis (Brown *et al.* 1997). It has been proposed that oxidative modifications of LDL increase its atherogenicity and that the resulting oxidized LDL, and not native LDL, plays a pivotal role in the development of foam cells of the fatty acid streak and its progression to more mature forms of the lesion. The oxidative changes in the modified LDL are associated with significant changes in the levels of oxysterols. In reviewing the general area of oxidized LDL, Steinberg *et al.* (1989) briefly noted that "LDL cholesterol is also oxidized during LDL modification, which could enhance its cytotoxicity and atherogenicity."

Of the oxysterols, 25-hydroxycholesterol (25-OHC) is the most extensively studied. 25-OHC is of interest because it has been described as one of the most toxic and atherogenic oxysterols (Smith and Johnson 1989; Zhou *et al.* 1993). It is an endogenous compound which is present in a diet containing cholesterol (Csallany *et al.*

1989; Hodis *et al.* 1991). 25-OHC has been found in human atheromatous plaques and aortic tissues (Hodis *et al.* 1991). It disrupts endothelial cell barrier function, disorganizes cytoskeletal protein and inhibits gap junctional communication. 25-OHC is known to modulate a number of biochemical steps involved with cholesterol metabolism. For example, 25-OHC down-regulates the cholesterol biosynthetic enzyme, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCoAR) (Smith and Johnson 1989), reduces transcription of LDL receptor (Sudhof *et al.* 1987) and stimulates cellular cholesterol esterification by the activation of acetyl-CoA: cholesterol acetyltransferase (ACAT) (Brown *et al.* 1975; Zhang *et al.* 1990). 25-OHC down regulates HMGCoAR and the LDL receptor by blocking the proteolytic activation of 2 sterol regulated transcriptional factors (Yang *et al.* 1995). Therefore, 25-hydroxycholesterol is commonly used as a model oxysterol on a wide variety of studies in cultured mammalian cells.

Apoptosis and Oxysterols

Cytotoxicity of oxysterols to many cell types has been widely reported including vascular cells such as endothelial cells (Ramasamy *et al.* 1992), macrophages (Aupeix *et al.* 1995; Clare *et al.* 1995), SMC (Peng *et al.*, 1979; Hughes *et al.* 1994), and lymphocytes (Christ *et al.* 1993). Death of any of these cell types stimulates atherogenesis. A loss of barrier function and exposure of a pro-coagulant surface through retraction and death of endothelial cells caused by oxysterols have been observed *in vivo* (Peng *et al.* 1985) and *in vitro* (Ramasamy *et al.* 1992). The macrophage death mediated by oxysterols has been suggested to contribute to the formation of the necrotic lipid core of advanced lesion (Ball *et al.* 1995). SMC death is associated with aneurysm development (Mayr and Xu 2001).

A number of studies have concerned the cytotoxicity of various oxygenated sterols. Recently, the cytotoxicity of oxysterols has been partly attributed to induction of apoptosis. There is some evidence for oxysterol-stimulated DNA fragmentation (Christ *et al.* 1993; Aupeix *et al.* 1995; Nishio and Watanabe 1996; Ares *et al.* 1997), decreased expression of Bcl-2 (an inhibitor of apoptosis) (Nishio and Watanabe 1996), activation of the protease caspase 3 (a key mediator of apoptotic cell death) (Ares *et al.* 1997), while caspase 3 inhibition partially blocked apoptosis induced by oxysterol (Nishio and Watanabe 1996; Harada *et al.* 1997). Oxysterols can induce apoptosis in a variety of cell types (Review: Schroepfer 2000). A model compound, 25-hydroxycholesterol, has been shown to induce apoptosis in monocyte-macrophage (Aupeix *et al.* 1995; Harada *et al.* 1997) and lymphoid cell lines (Bansal *et al.* 1991; Christ *et al.* 1993; Ayala-Torres *et al.* 1997) in the range of 1-10 μ M. Related work by Ayala-Torres *et al.* (1997) concerning the effects of 25-OHC on apoptosis in CEM cells. Bansal *et al.* (1991) also reported that 25-OHC induced apoptosis, as measured by DNA fragmentation, in human leukemia cell lines (CEM-C7). Other study found that 25-OHC blocked the growth of cells in the postmitotic half of the G1 phase of the cell cycle (Larsson and Zetterberg 1986). Another model oxysterol, 7-ketocholesterol, has also been shown to induce apoptosis in vascular endothelial and smooth muscle cell (Lizard *et al.* 1996, 1999). These apoptotic cells are observed *in vivo* in atherosclerotic lesions.

The relative toxicities of oxysterols to cells *in vitro* depend very much on the conditions under which exposure is conducted, the cell type and even the species. For example, the concentration of oxysterol required to achieve detectable toxicity is higher in the presence of serum or lipoproteins than in serum-free media; this is probably due to the capacity of lipoproteins to absorb oxysterols and reduce their effective concentration as well as direct antagonism of oxysterol toxicity by cholesterol (Clare *et al.* 1995; Colles *et al.* 1996; Nishio *et al.* 1996).

Signal Transduction of Apoptosis Induced by OxLDL/Oxysterols

Apoptosis is an active process triggered by many cytotoxic stress stimuli. Different cytotoxic stress stimuli, such as tumor necrosis factor, γ radiation, UV radiation, hydrogen peroxide, and growth factor removal, induce apoptosis in many cell types by both unique and common mechanisms (Wertz and Hanley 1996; Escargueil-Blanc *et al.* 1997). For example, Fas and tumor necrosis factor receptor family members transduce the signal of apoptosis through death domain-containing molecules, such as FADD, whereas many other agents induce apoptosis by pathways that do not involve protein molecules with a death domain.

Apoptosis encompasses at least two stages, including the early stage of signal initiation (signaling phase) and the later stage of final execution (execution phase) (Earnshaw 1995). The signaling phase relates to the events leading up to the regulation of apoptotic specific proteins such as the caspase family. The execution phase of apoptosis is comprised of those processes that commit cells to apoptotic death. There are many signaling pathways that converge on to other pathways leading to the commitment to die (execution phase). Once a cell commits to die, the execution phase of cell death is similar even though the pathway by which it gets to this point may differ. The caspase family has been believed to play pivotal roles in the execution of apoptosis (Chang and Yang 2000).

It has been proposed that oxLDL/oxysterol might be involved in signal transduction during oxLDL/oxysterol-induced apoptosis. Clarification of the molecular mechanism of signaling transduction that regulates oxLDL/oxysterol induced apoptosis may help in the design of a new strategy for treatment of atherosclerosis and its major complication, the acute vascular syndromes. Progress in basic research on signaling of oxysterol/oxLDL to the execution phase provides the opportunity to attack at least some

signal-transduction targets involved in oxLDL/oxysterol-induced apoptosis. Although the intracellular pathway by which oxysterol/oxLDL induces apoptosis has not been completely clarified, there have been a few relevant studies on this pathway.

Increases in Cytosolic Ca²⁺ for Signal Transduction of Oxysterol/OxLDL-Induced Apoptosis

Ca²⁺ is recognized as an important regulator of apoptosis (Marks 1997; McConkey and Orrenius 1997; Distelhorst and Dubyak 1998). The cytoplasmic Ca²⁺ concentration is maintained at ~100 nM in resting cells by relatively impermeable cell membranes, active extrusion of Ca²⁺ from the cell by plasma membrane Ca²⁺-ATPases, plasma membrane Na⁺/Ca²⁺ exchangers, and active uptake of cytosolic Ca²⁺ into the endoplasmic reticulum (ER) by distinct Ca²⁺-ATPases. In contrast, the concentration of Ca²⁺ in the extracellular milieu and in the ER is much higher (in the millimolar range). Evidence for involvement of Ca²⁺ influx into the cytosol as a triggering event for apoptosis has come from studies with specific Ca²⁺ channel blockers that abrogate apoptosis in regressing prostate following testosterone withdrawal (Martikainen and Isaacs 1990). Other support for the involvement of Ca²⁺ in apoptosis comes from the observation that agents that directly mobilize Ca²⁺ (e.g. Ca²⁺ ionophores or the sarcoplasmic reticulum Ca²⁺-ATPase pump inhibitor, thapsigargin, TG) can trigger apoptosis in diverse cell types (Jiang *et al.* 1994; Kaneko and Tsukamoto 1994; Levick *et al.* 1995). Inhibition of the sarcoplasmic reticulum Ca²⁺-ATPase pump by TG causes a transient increase in cytoplasmic Ca²⁺ from ER Ca²⁺ stores, and a later influx of Ca²⁺ from the extracellular milieu, leading to the induction of apoptotic cell death (Kaneko and Tsukamoto 1994; McColl *et al.* 1998; Jackisch *et al.* 2000). Consequently, emptying of intracellular Ca²⁺ stores may trigger apoptosis by disrupting the intracellular architecture and allowing key elements of the effector machinery (e.g. Apaf-1) to gain access to their

substrates (e.g. caspase 9). Ca^{2+} has also been shown to be necessary for apoptotic endonuclease activation, eliciting DNA cleavage after many cellular insults (Gaido and Cidlowski 1991; Urbano *et al.* 1998; Yakovlev *et al.* 2000). Buffering intracellular Ca^{2+} released from stored Ca^{2+} pools (e.g. ER) with BAPTA-AM, or removal of extracellular Ca^{2+} with EGTA, can protect cells against apoptosis (McConkey *et al.* 1989; Srivastava *et al.* 1999). Therefore, increases in intracellular Ca^{2+} levels appear to be important cell death signals in human cancer cells that might be exploited for anti-tumor therapy.

Oxidized LDL has been reported to induce a rise in the level of intracellular Ca^{2+} that was followed by activation of proteolysis and DNA fragmentation in human lymphoblastoid cells during the induction of necrosis and apoptosis by oxLDL (Escargueil-Blanc *et al.* 1994; Ares *et al.* 1997; Escargueil-Blanc *et al.* 1997;). Measurement of cytosolic Ca^{2+} with the permeable dye fura2, indicates that cytosolic Ca^{2+} increases in response to treatment with oxLDL but that this Ca^{2+} influx can be blocked by chelation of the Ca^{2+} with extracellular EGTA. This treatment with EGTA also prevents apoptosis (Escargueil-Blanc *et al.* 1998). Other studies (Escargueil-Blanc *et al.* 1997) demonstrate that the calcium channel blockers nifedipine and nisoldipine block oxLDL induced apoptosis as does the endonuclease inhibitor, aurintricarboxylic acid. This observation is interpreted as being consistent with the apoptosis being mediated by a Ca^{2+} dependent endonuclease. Others (McConkey *et al.* 1989), had previously implicated increased intracellular calcium in glucocorticoid induced apoptosis of thymocytes. In 1985, Boissonneault and Heiniger reported that 25-OHC caused a stimulation of the uptake of Ca^{2+} into P815 mastocytoma cells. The stimulation of the uptake of Ca^{2+} by 25-OHC was dependent on the level of 25-OHC. Furthermore, Ares *et al.* demonstrated that 25-OHC-induced apoptosis could be blocked by two Ca^{2+} channel blockers, verapamil and nifedipine in human aortic smooth muscle cells (Ares *et al.* 1997).

Ca^{2+} may act as a signal for apoptosis by directly activating key proapoptotic enzymes (e.g. calpain, cPLA₂). Many intracellular signaling events are triggered by transient changes in the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Intracellular Ca^{2+} increases initiate gene expression and cell cycle progression, but also can activate degradative processes in apoptosis. Prolonged high $[\text{Ca}^{2+}]_i$ activates nucleases that cleave DNA and degrade cell chromatin. Ca^{2+} promotes DNA digestion by direct stimulation of endonucleases, or indirectly by its activation of Ca^{2+} -dependent protease, phosphatases, and phospholipases, resulting in a loss of chromatin structural integrity (Nicotera *et al.* 1994). The study was intriguing with regard to the possible role of Ca^{2+} as a signal in the 25-OHC-induced apoptosis since high frequency Ca^{2+} oscillations were observed within minutes of addition of 25-OHC (Ares *et al.* 1997). Calcium signaling has been observed to occur with two modalities: amplitude modulation and frequency modulation (Berridge 1997). This refers specifically to signaling mediated by the magnitude and duration of a change in intracellular Ca^{2+} versus a change in the frequency of oscillation of intracellular Ca^{2+} . Both modalities of regulation have been observed and could be consistent with the literature observations on oxLDL and 25-OHC.

Involvement of Calcium Dependent Cytosolic Phospholipase A₂ (cPLA₂) Activation and Arachidonic Acid (AA) Release in Apoptosis Pathway

General interest for phospholipases A₂ (PLA₂s) comes mainly from their role in the liberation of arachidonic acid. This enzyme preferentially hydrolyzes phospholipids containing arachidonate at the sn-2 position, thus providing free arachidonic acid (AA) for the biosynthesis of eicosanoids, which are an important class of lipid mediators that modulate a wide variety of physiological functions. Mammalian PLA₂s (Dennis 1994) are a large superfamily of enzymes with distinct structural and biochemical characteristics

and different roles in signal transduction and general lipid metabolism (Dennis 1994, 1997; Murakami *et al.* 1997). According to an updated classification, PLA₂s can be subdivided into several groups based upon their structures and enzymatic characteristics. The best knowns of these are Ca²⁺-dependent secretory PLA₂ (sPLA₂), Ca²⁺-independent PLA₂ (iPLA₂), and Ca²⁺-dependent cytosolic PLA₂ (cPLA₂) (Murakami *et al.* 1998; Chaminade *et al.* 1999).

sPLA₂s are low molecular mass (~14 kDa) enzymes which are cysteine-rich, secreted proteins that require millimolar concentrations of calcium for activity. They can liberate AA, but they exhibit little preference for sn-2 AA (Dennis 1997). Therefore, based on these properties it appears unlikely that the role of this type of PLA₂ is to initiate AA release from inside the cell.

iPLA₂ was initially recognized in heart cytosol as a 40 kDa protein which has no calcium requirement for its enzymatic activity (Hazen *et al.* 1993; Chaminade *et al.* 1999). It exhibits a substrate preference for phospholipids with AA at the sn-2 position. iPLA₂ has been proposed to participate in fatty acid release associated with phospholipid remodeling and to play a minimal role in signal transduction (Ackermann *et al.* 1995; Balsinde and Dennis 1997; Balsinde *et al.* 1997).

cPLA₂ is a ubiquitously distributed 85-kDa enzyme with no sequence homology to any of the other PLA₂s (Clark *et al.* 1991; Sharp *et al.* 1991; Clark *et al.* 1995; Kramer and Sharp 1997; Murakami *et al.* 1997;). This enzyme preferentially hydrolyzes glycerolphospholipids containing arachidonate at the sn-2 position and is regulated by physiological levels of intracellular calcium concentration (Clark *et al.* 1991; Kramer *et al.* 1991). It is activated by 2 complementary events: calcium-dependent translocation of the protein to the membrane, and phosphorylation, which promotes increased activity of the enzyme. The N-terminal calcium-phospholipid binding domain (CALB domain) similar to the C2 domain presenting in protein kinase C (Perisic *et al.* 1998) is

responsible for Ca^{2+} -dependent translocation of cPLA₂ from the cytosol to perinuclear and endoplasmic reticular membrane. Interestingly, a number of enzymes involved in eicosanoid metabolism, such as prostaglandin endoperoxide synthase 1 and 2 (the two cyclooxygenase (COX)) and 5-lipoxygenase also localize to the nuclear envelope and endoplasmic reticulum (Glover *et al.* 1995; Schievella *et al.* 1995; Peters-Golden *et al.* 1996; Serhan *et al.* 1996). In addition to calcium, the activation of cPLA₂ by some agonists requires phosphorylation of the enzyme. cPLA₂ contains 2 serine residues (505 and 727) probably phosphorylated by various mitogen-activated protein kinases (MAP kinases). These enzymatic features of cPLA₂ are consistent with its role in immediate eicosanoid biosynthesis occurring within minutes of stimulation, which is usually accompanied by rapid and transient cytoplasmic Ca^{2+} mobilization and mitogen-activated protein kinase activation. The AA thus liberated is supplied to constitutive COX-1 and 5-lipoxygenase to be converted into prostanoids and leukotrienes, respectively. cPLA₂ has also been implicated in delayed, COX-2 dependent prostanoid generation lasting for hours despite the absence of Ca^{2+} signal in this setting (Hayakawa *et al.* 1993; Lin *et al.* 1993). Experiments with mice in which the cPLA₂ gene has been disrupted clearly demonstrate an obligatory role of cPLA₂ in eicosanoid production (Fujishima *et al.* 1999). These studies indicate that cPLA₂ is an important mediator of stimulus-induced AA release and subsequent eicosanoid synthesis.

PLA₂s are involved in the induction of apoptosis of a number of cell lines (Voelkel-Johnson *et al.* 1996; Wissing *et al.* 1997; Atsumi *et al.* 1998; Wu *et al.* 1998; Duan *et al.* 2001). Evidence was given for a direct involvement of cPLA₂ in apoptosis promoted by tumor necrosis factor α (TNF α) in a mechanism implicating enzyme activation via caspase-mediated proteolysis (Thorne *et al.* 1996; Dimitrov *et al.* 1997; Foghsgaard and Jaattela 1997; Wissing *et al.* 1997; Wu *et al.* 1998). It has been reported that apoptosis induced by tumor necrosis factor (TNF) in a number of different

cell types depended on the activity of cPLA₂, which catalyzes the release of arachidonic acid from the sn-2 position of membrane phospholipids (Wolf and Laster 1999). This apoptosis concomitant arachidonic acid release is abrogated by group IV cPLA₂ inhibitors (methyl arachidonyl fluorophosphate and methyl trifluoromethyl ketone), but not by inhibitors of group VI Ca²⁺-independent (iPLA₂; bromoenol lactone) or of secretory low molecular mass PLA₂ (Duan *et al.* 2001).

AA is emerging as a very important player in the pathways to cell death. Nonsteroidal anti-inflammatory drugs, which are among the few agents that can effectively prevent neoplasias (Baron and Sandler 2000), induce apoptosis through elevation of the intracellular levels of AA, and their effects can be mimicked by the addition of exogenous AA (Chan *et al.* 1998). Wolf and Laster (1999) also demonstrated that treatment of cells, under appropriate conditions, with arachidonate could be shown to specifically induce apoptosis presumably by mimicking the metabolic consequences of cPLA₂ activation. Furthermore, apoptosis induction by AA has been found to involve activation of caspase-3, a process that is amplified by release of mitochondrial cytochrome c (Liu *et al.* 1996). Scorrano *et al.* (2001) reported that arachidonic acid caused a mitochondrial permeability transition (PT) *in situ* that was accompanied by cytochrome c release and rapidly followed by cell death.

OxLDL (100 µM), but not native LDL, has been reported to markedly increase cytosolic phospholipase A₂ activity and arachidonic acid release in a time- and dose-dependent manner. AACOCF₃, a potent cPLA₂ inhibitor strongly inhibited the stimulated AA release (Lupo *et al.* 2001). Other studies demonstrated that oxysterols could also stimulate arachidonic acid release. The stimulation of arachidonate release and the formation of the prostaglandins PGE₂ and PGF_{2α} by oxysterol (25-OHC) were only observed in the presence of FCS (Lahoua *et al.* 1988). On the basis of further studies with 25-OHC, Lahoua *et al.* suggested that stimulation by the oxysterol of arachidonate

release and PGE₂ biosynthesis involved activation of phospholipase A₂ and action of 25-OHC in these processes might involve effects on Ca²⁺ flux (Lahoua *et al.* 1989, 1991).

Peroxisome Proliferator-Activated Receptors Gamma (PPAR_γ) Signal Transduction Pathway

Peroxisome proliferator-activated receptors gamma (PPAR_γ) signal transduction pathway is one reported apoptosis pathway related to a particular AA metabolites-prostaglandin. It is observed that peroxisome proliferator-activated receptors (PPARs) are activated by arachidonic acid metabolites. PPARs are a group of ligand-activated transcription factors which function as regulators of lipid and lipoprotein metabolism and glucose homeostasis and influence cellular proliferation, differentiation and apoptosis (Mangelsdorf and Evans 1995; Schoonjans *et al.* 1997). Among three subtypes (α, β, and γ) of PPAR, PPAR_α is highly expressed in liver, muscle, kidney, and heart, where it stimulates the beta-oxidative degradation of fatty acids (Isseman and Green 1990; Braissant *et al.* 1996). A recent study revealed that leukotriene B₄ is a ligand for PPAR_α (Devchand *et al.* 1996). PPAR_γ is predominantly expressed in intestine and adipose tissue (Tontonoz *et al.* 1994), where it triggers adipocyte differentiation, glucose metabolism, inflammatory responses, macrophage differentiation, and energy homeostasis by regulating the expression of genes (Schoonjans *et al.* 1997; Jiang *et al.* 1998; Nagy *et al.* 1998; Ricote *et al.* 1998; Spiegelman 1998; Tontonoz *et al.* 1998). PPAR_γ is selectively activated by the prostaglandin J₂ series including Δ¹²-prostaglandin J₂ and 15-deoxyl-Δ^{12,14}-prostaglandin J₂ (Kliwer and Willson 1998; Spiegelman 1998). Recently, the expression of PPAR_α and PPAR_γ was also reported in cells of the vascular wall, such as monocyte/macrophages, endothelial and smooth muscle cells (Chinetti *et al.* 1998).

PPARs behave as ligand-dependent transcription factors, which, upon heterodimerization with the 9-cis-retinoic acid receptor, bind to specific response elements in the promoter of specific target genes termed peroxisome proliferator-response element (PPRE), thus regulating the expression of target genes. Most PPREs identified to date reside in genes involved in intra- and extra-cellular lipid and lipoprotein metabolism (Schoonjans *et al.* 1997). Furthermore, the macrophage scavenger receptor CD36, which also functions as a fatty acid transporter in adipose tissue (Abumrad *et al.* 1993), has been identified as a PPAR γ target gene in macrophage (Tontonoz *et al.* 1998). It has been observed that activation of PPAR γ may favor foam cell formation through induction of scavenger receptors in those macrophages (Nagy *et al.* 1998). Therefore, PPARs play a role in metabolic conditions leading to atherosclerosis development. In addition, PPARs also regulate genes involved in inflammatory control, since PPAR γ activators have been shown to inhibit the activation of macrophages by interfering with the transcriptional induction of genes such as TNF α , iNOS, and gelatinase B by inflammatory agents (Jiang *et al.* 1998; Ricote *et al.* 1998).

PPARs have also been demonstrated to play a role in the control of cell apoptosis. Activation of PPAR γ has been reported to block angiogenesis of endothelial cells and suppresses transcriptional activation of COX-2 (Bishop-Bailey *et al.* 1999). A natural PPAR γ ligand, prostaglandin 15d-PGJ₂ (Forman *et al.* 1995; Kliewer *et al.* 1995), has been shown to be the inducer of apoptosis in a number of cell types including a variety of tumor cell lines (Keelan *et al.* 1999; Takahashi *et al.* 1999; Clay *et al.* 2001), vascular endothelial cells (Bishop-Bailey and Hla 1999), and monocyte-derived macrophage (Chinetti *et al.* 1998). In all these cell types, synthetic ligands of PPAR γ including antidiabetic thiazolidinediones (Lehmann *et al.* 1995) also are inducers of apoptosis, consistent with the hypothesis that is the activity of 15d-PGJ₂ as a PPAR γ ligand which makes it cytotoxic.

The cyclopentenone 15d-PGJ₂ is the cyclooxygenase metabolites of arachidonic acid. It is a terminal derivative of prostaglandin and may derive from PGD₂ (Li *et al.* 2001), which is first identified as an inducer of adipogenesis through activation of PPAR γ . Apoptotic and growth inhibitory properties of 15d-PGJ₂ suggest that this cyclopentenone prostaglandin may be a negative regulator of cell proliferation. Oxysterols alter the production of eicosanoids in various cells. It has been reported that 25-hydroxycholesterol treatment of arterial endothelial and smooth muscle cells produces a dramatic increase in the incorporation of labeled arachidonic acid into total prostaglandins (Wohlfeil and Campell 1997, 1999) and this is due, at least in part, to upregulation of prostaglandin G/H synthase-2 (COX-2). Observations consistent with activation of calcium uptake and subsequent enhancement of prostacyclin synthesis by 25-hydroxycholesterol have also previously been reported (Kawamura and Kummerow 1992).

Caspase: Executioner and Undertaker of OxLDL/Oxysterols Induced Apoptosis

The caspase family plays pivotal roles in the execution of apoptosis. Almost all known agents that induce apoptosis share the activation of caspases and the execution phase of the death program (Cohen 1997; Nicholson and Thornberry 1997; Salvesen and Dixit 1997). Caspases are cysteine proteases that cleave after aspartic acid residues. The term caspase reflects the catalytic properties of these enzymes, the “c” denotes their cysteine protease mechanism and “aspase” refers to their ability to cleave after aspartic acid residues. The caspase family was originally discovered following a search for mammalian homologues of ced-3, a cell death gene described in the nematode worm *C.elegans*. The first mammalian caspase identified was ICE (interleukin-1 β converting enzyme), now known as caspase-1. Numerous other caspases have been discovered and each has been given a variety of names: caspase-

1 (ICE), caspase-2 (ICH-1, Nedd-2), caspase-3 (CPP32, Yama, apopain), caspase-4 (TX, ICH-2, ICERel-II, also homologous to murine caspase-11), caspase-5 (ICERel-III, TY), caspase-6 (Mch-2), caspase-7 (Mch-3, ICE-LAP, CMH-1), caspase-8 (MACH, FLICE, Mch-5), caspase-9 (Apaf-3, ICE-LAP6, Mch-6), caspase-10 (Mch-4), caspase-13 (ERICE), and caspase-14 (MICE) (Chang and Yang 2000).

Sequence analysis and x-ray crystallography data suggest that all caspases share a common structure (Van de Craen *et al.* 1997; Wolf and Green 1999). Caspases are synthesized as single-chain inactive zymogens that await activation within the cell. Each zymogen contains an N-terminal prodomain, a large subunit containing the active site cysteine within a conserved QACXG motif, and a C-terminal small subunit. An aspartate cleavage site separates the prodomain from the large subunit, and an interdomain linker containing one or two aspartate cleavage sites separates the large and small subunits. Activation accompanies proteolysis of the interdomain linker and usually results in subsequent removal of the prodomain. The large subunit and small subunit come together in a heterodimer. The 2 heterodimers come together to form a tetramer. The crystal structures of active caspase reveal a tetramer in the active, processed state (Wolf and Green 1999). The heterodimers each contain an active site composed of residues from both the small and large subunits. Each active site contains a positively charged S1 subsite that binds the substrate's negatively charged P1 aspartate (Wolf and Green 1999). This S1 binding site is highly conserved; therefore, all caspases cleave solely after aspartate residues.

Singularly important is that caspase zymogens are themselves substrates for caspases, such that some are able to activate others in a hierarchical relationship. Thus, pathways exist to transmit signals via sequential caspase activation, and this event has been most extensively examined in apoptosis. It is relatively easy to imagine that the caspases operating at the bottom of the pathway are activated by the ones

above. Therefore, caspases can be divided into two main classes: initiator and effector caspases. Initiator caspases are the upstream activators of the effector caspases. Effector caspases are the executioners in the cell. They cleave the proteins that actually induce apoptosis in the cell. These cleavages lead to the morphological features: membrane blebbing, cytoplasmic and nuclear condensation, DNA fragmentation, and the formation of apoptotic bodies. Caspase substrate specificity, prodomain length, and prodomain sequence determine caspase function (Wolf and Green 1999). Caspases can be grouped according to their substrate specificities, which are largely determined by the amino acids preceding the cleavage site aspartic acid residue. The substrate specificity of one group of caspases that includes caspases-8 and -9 is (V/L)EXD, a site similar to that found in caspase proenzymes (Thornberry *et al.* 1997). Therefore, these caspases may function as initiators of a proteolytic cascade by activating pro-caspases to amplify a death signal. In contrast, the substrate specificity of a 2nd group of caspases, which includes caspase-3 is DEXD, a cleavage site that found in many target proteins that are cleaved during the effector phase of cell death (Thornberry *et al.* 1997). Therefore, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins in response to many apoptotic stimuli (Kaufmann *et al.* 1993; Casciola-Rosen *et al.* 1994, 1995; Wang and Keiser 1998). Cells from mice deficient in caspase-3 show stimulus-specific defects in apoptosis that include delayed kinetics, incomplete chromatin condensation, and the absence of DNA fragmentation (Kuida *et al.* 1996).

A critical issue is how caspases become initially activated, which seems to be an irreversible commitment towards death. It seems that aggregation of some pro-caspases (those with large pro-domains) allows them to autoactivate. Recent experiments make it clear that mitochondria are involved in one major pathway involving activation of pro-caspase-9, which is called as the death receptor-independent,

"intrinsic" pathway (Reed 1997; Green and Reed 1998). Other experiments show that ligands crosslinking death receptors, such as Fas, trigger formation of a cytoplasmic complex in which pro-caspase-8 is aggregated and activated. This process is called the death receptor-dependent, "extrinsic" pathway (Li *et al.* 1998; Luo *et al.* 1998; Gross *et al.* 1999). In both cases, once initiator caspase is activated, these activated caspases transactivate other procaspases, providing the opportunity for cascade amplification and positive feedback.

The death receptor-dependent, "extrinsic" pathway involves trimerization of death receptors such as CD95/FAS/APO-1 or TNF receptor 1 by binding of their respective ligands, which leads to recruitment of the activator caspase 8 via adapter molecules like FADD and TRADD and to its autoactivation (Nagata 1997). Activated caspase 8 can directly cleave and activate the effector caspase (Boldin *et al.* 1996; Muzio *et al.* 1996), or it can cleave the Bcl-2 family member Bid to induce the release of mitochondrial cytochrome C, which also leads to activation of effector caspases via oligomerization with Apaf-1 and caspase 9 in the presence of ATP (Li *et al.* 1998; Luo *et al.* 1998; Gross *et al.* 1999).

In contrast, the death receptor-independent, "intrinsic" pathway is directly activated by a death signal, leading to the release of cytochrome C from the mitochondrial intermembrane space into the cytosol, which then, in the presence of ATP, facilitates oligomerization and activation of Apaf-1 and caspase 9, leading to activation of caspase 3 and other effector caspases (Green and Reed 1998). The release of cytochrome C is regulated by the various pro- and anti-apoptotic members of the Bcl-2 family (Reed 1997; Jurgensmeier *et al.* 1998; Finucane *et al.* 1999; Schuler *et al.* 2000). In a healthy cell, the outer membranes of its mitochondria express the protein Bcl-2 on their surface. Bcl-2 is bound to a molecule of the protein Apaf-1. Internal damage in the cell causes Bcl-2 to release Apaf-1 and to no longer keep cytochrome c

from leaking out of the mitochondria. The released cytochrome c and Apaf-1 bind to molecules of caspase 9 which results the formation of complex of cytochrome c, Apaf-1, and caspase 9. Caspase 9 is one of a family of over a dozen caspases. It cleaves and, in so doing, activates other caspases. The sequential activation of one caspase by another creates an expanding cascade of proteolytic activity that leads to digestion of structural proteins in the cytoplasm, degradation of chromosomal DNA and death of the cell (Li *et al.* 1997; Zou *et al.* 1997; Hu *et al.* 1999; Saleh *et al.* 1999).

It has been reported that oxLDL/oxysterol can activate caspases and the apoptosis induced by oxLDL/oxysterol could be blocked by caspase inhibitors (Farber *et al.* 1999; Vicca *et al.* 2000) in various vascular cells. Activation of caspases by oxysterols/oxLDL has been reported through both the death receptor-independent and – dependent pathway. Lee and Chau (2001) reported that Fas/FasL death pathway is activated and responsible, at least in part, for apoptotic death in vascular SMCs upon exposure to oxLDL. They demonstrated that the expression of Fas and Fas ligand (FasL) was substantially upregulated by oxLDL in cultured vascular smooth muscle cells (SMCs). Two oxysterols, 7 β -hydroxycholesterol and 25-hydroxycholesterol, were also effective in inducing the expression of Fas, Fas ligand, and apoptosis. Incubation of cells with FasL-neutralizing antibody inhibited the oxLDL-induced cell death up to 50%. Caspase 8 and caspase 3 activities were induced time dependently in SMCs following oxLDL treatment. Other researchers (Sata and Walsh 1998; Napoli *et al.* 2000) also reported attenuation of apoptotic induction by oxLDL in human coronary artery endothelial cells and smooth muscle cells after blockage of the Fas pathway with Fas ligand neutralizing antibody or cellular caspase inhibitor FLICE-inhibitory protein.

The Bcl-2 protein family/mitochondrial mediated cytochrome C release pathway has also been reported in oxysterol/oxLDL-induced apoptosis. It has been demonstrated that oxLDL treatment triggered the release of cytochrome C from the

mitochondria into the cytosol, indicating disturbance of the mitochondrial membrane (Walter *et al.* 1998). Another study also reported that 7-ketocholesterol-induced apoptosis was associated with an important release of cytochrome c into the cytosol and a noticeable degradation of procaspase-8 (Lizard *et al.* 1998).

Specific Aims

Apoptotic cells induced by oxLDL/oxysterol have been observed *in vivo* in atherosclerotic lesions. The presence of apoptotic cells in atherosclerotic lesions could have potential clinical implications for atherogenesis and contributes to the instability of the lesion. According to these considerations, characterization of cell death induced by oxysterols has some pathophysiological and therapeutic ramifications for the understanding of the atherosclerosis process as well as for treatment of the lesions. However, it is not clear how oxLDL/oxysterol induces apoptosis in vascular cells. The purpose of our study is to investigate the signaling pathway of apoptosis induced by oxidized LDL. First, we will create a simple model system that is easier to work on. Then, we will use this model to examine the molecular pathways leading to apoptotic cell death including signal transduction and execution phases of apoptosis.

CHAPTER 2

MATERIALS AND METHODS

Materials

CHO-K1 cells were purchased from the American Type Culture Collection (Manassas, VA). All cell culture reagents were obtained from Life Technologies, Inc (Rockville, MD). 25-Hydroxycholesterol was from Steraloids Inc. (Wilton, NH). $^{45}\text{Ca}^{2+}$ was from Amersham Pharmacia Biotech. Dil and fluorescein isothiocyanate- or Texas Red-conjugated secondary antibodies were from Molecular Probes, Inc. (Eugene, OR). Anti-CD36 monoclonal antibody was from Pharmingen (San Diego, CA). Horseradish peroxidase-conjugated goat anti-mouse IgM and IgG and the micro-BCA protein assay kit were from Pierce. Ac-DEVD-AMC, Ac-DEVD-CHO, and Ac-IETD-AFC were from pharmingen (San Diego, CA). Ac-LEHD-AFC was from Enzyme System Products. Anti-cytochrome C and anti-PARP monoclonal antibody were also from pharmingen (San Diego, CA). TUNEL assay kit was from Boehringer. 5,8,11,14-Eicosatetraynoic acid (ETYA), Bromoenol lactone (BEL), Nifedipine, and 15-deoxy- $\Delta^{12,14}$ prostaglandin J_2 (15d-PG J_2) were from Biomol (Plymouth Meeting, PA). Arachidonyl trifluoromethyl ketone (AACOCF $_3$) was from Alexis Corporation (San Diego, CA). [5, 6, 8, 9, 11, 12,14,15- ^3H] arachidonic acid (200 Ci/mmol) was from American Radiolabeled Chemical, Inc (St. Louis, MO). The plasmid pEGFP-N3 carrying human cPLA $_2$ cDNA was generously provided by Dr. Chistina C. Leslie (Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Dever, Colorado). The plasmid pcDNA3 carrying human CD36FLAG cDNA was generously provided by Dr. D. M. Lublin (Department of Pathology, Washington University School of Medicine, St. Louis, MO). SREBP2-GFP constructs were kindly provided by Dr. T. F. Osborne (Department of Molecular Biology and Biochemistry, University of California, Irvine, CA).

The plasmid of peroxisome proliferator response element (PPRE) luciferase reporter (AOX)3 TK-Luc was a kind gift of Dr. C Glass, UCSD. All organic solvents were high-performance liquid chromatography grade and were purchased from Fisher. Mammalian transfection kit were from Stratagene (Stratagene, La Jolla, CA). Promega firefly luciferase reporter assay kit were purchased from Promega.

Methods

Cell Culture

CHO-K1 cells were purchased from the American Type Culture Collection (Manassas, VA) and were grown in Ham's F-12 medium containing 5 % fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin (F12FC5) at 37 °C and 5 % CO₂. All cell reagents were obtained from Life Technologies, Inc (Rockville, MD).

Preparation of Modified LDL

LDL ($1.019 < d < 1.063$) was prepared from normal human serum by sequential ultracentrifugation as described previously (Rusinol *et al.* 2000). Extensively oxidized LDL was prepared by incubation with CuCl₂ as described previously (Brown *et al.*, 1997). Oxidation of LDL was evaluated by agarose gel electrophoresis. The oxLDL had 3-5-fold higher RF values on agarose gel electrophoresis compared with native LDL.

Dil-labeled oxLDL was prepared as described elsewhere (Pitas *et al.* 1981). Essentially, oxLDL was incubated with the Dil probe in human lipoprotein-deficient serum for 12 h at 37 °C, using the following relative amounts: 300 µl (300 µg) of Dil, 3 mg of lipoprotein lipid, 2 ml of lipoprotein-deficient serum. Subsequently, the labeled lipoproteins are reisolated by ultracentrifugation for 2.5 h at 99,000 rpm in a TL-100 centrifuge. Labeled lipoproteins were stored at 4 °C in the dark and used within 2 weeks after their preparation.

Cytotoxicity Assays (Surviving Assay)

CHO-K1 cells were seeded at a density of 500 or 1000 cells/35- or 60-mm dish in F12FC5 on day 0. On day 1, the cells were rinsed with phosphate-buffered saline (PBS) twice and then fed either Nutridoma-SP (1% in Ham's F-12) or F12FC5 containing oxysterols or oxLDL or various inhibitors with or without 25-hydroxycholesterol or 15-deoxy- $\Delta^{12,14}$ prostaglandin J_2 (15d-PG J_2), as described in the figure legends. Following incubation, cells were fed fresh F12FC5 and allowed to grow for 5 days. The surviving colonies were then fixed and stained with crystal violet as described (Sinensky *et al.* 1980).

Isolation of Permanent Transfectants Expressing CD36

CHO-K1 cells were grown in Ham's F-12 medium containing 5% fetal calf serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin (F12FC5) at 37 °C and 5% CO $_2$ and transfected with pcDNA3, carrying human CD36FLAG cDNA and neomycin resistance, by the lipofectamine method using a mammalian transfection kit (Stratagene, La Jolla, CA). The neomycin-resistant cells were selected using 500 μ g/ml G418 (Life Technologies, Inc.) in F12FC5. Resistant colonies were isolated and assayed for the Dil-oxLDL binding as described below. Colonies expressing CD36 activity were expanded and maintained in F12FC5 containing 500 μ g/ml G418.

Detection of CD36 in CHO/CD36

Transfected cells were grown overnight on glass coverslips and then incubated with 10 μ g/ml Dil-oxLDL for 1 h at 37 °C. After washing with PBS, cells were fixed in freshly prepared 4% formaldehyde in PBS and mounted. Cell association of Dil-labeled oxLDL was observed by fluorescence microscopy using a Texas Red filter cube.

Isolation of a 25-Hydroxycholesterol-Resistant CHO/CD36 Mutant

CHO/CD36 cells, plated at 3×10^6 cells per 100 mm plate and allow to attach for 4 h in F12FC5 medium, were mutagenized by incubation for 16 h in 10 ml culture medium containing 4 μ l of ethyl methane sulfonate (methanesulfonic acid ethyl ester). Cells were washed three times with PBS and refed with F12FC5. After 24 h incubation, one plate of cells were split into 2 plates (100 mm) and incubated in fresh culture medium for 36-48 h. The 25-hydroxycholesterol resistant mutant cells were selected using 3 μ g/ml 25-hydroxycholesterol in F12FC5. Resistant colonies were isolated. Colonies growing out in the presence of 25-OHC (3 μ g/ml) were expanded and maintained in F12FC5 containing 25-OHC (3 μ g/ml).

TUNEL (Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End Labeling) Assay

Cells (50,000) were plated on glass coverslips and incubated for different periods of time in F12FC5 with or without 10-50 μ g/ml oxLDL or 3 μ g/ml 25-OHC. After washing with PBS, the cells were fixed in 4% buffered paraformaldehyde for 30 min at room temperature. Coverslips were rinsed, and cells were permeabilized with 1% Triton X-100 in 100 mM sodium citrate buffer and incubated for 1 h at 37 °C with terminal deoxynucleotidyltransferase and fluorescein isothiocyanate-dUTP to label the fragmented DNA. After completion, coverslips were mounted in anti-fade mounting solution and observed under a Nikon Diaphot-200 microscope.

Detection of Fragmented DNA on Agarose Gels

Detection of oligonucleosomal DNA fragments was done as described previously (Meßmer *et al.* 1996). Basically, cells at an initial density of 5×10^5 /100-mm dish were cultured for 24 h in F12FC5 plus oxLDL or oxysterols and then harvested, lysed, and centrifuged to separate DNA fragments from intact chromatin. Supernatants were

precipitated overnight at 20 °C with 2 volumes of ethanol and centrifuged at $13,000 \times g$ for 15 min. Then pellets were incubated for 30 min at 37 °C in 500 µl of Tris-EDTA (TE) buffer supplemented with 100 µg/ml RNase A. Samples were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and once again with chloroform/isoamylalcohol (24:1). DNA was precipitated, and pellets were recovered by centrifugation ($13,000 \times g$, 15 min), air-dried, resuspended in 10 µl of TE buffer, supplemented with 2 µl of sample buffer (0.25% bromphenol blue, 30% glycerol), and electrophoretically separated on a 1.3% agarose gel. After electrophoresis, they were stained in ethidium bromide (1 µg/ml). Pictures were taken by UV transillumination.

Isolation of Permanent Transfectants Expressing SREBP2

CHO-K1 cells were grown in Ham's F-12 medium containing 5% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (F12FC5) at 37 °C and 5% CO₂ and transfected with 6 µg of the expression vector encoding a SREBP2 fusion protein (SREBP2-GFP) by the lipofectamine method using a mammalian transfection kit (Stratagene, La Jolla, CA). The neomycin-resistant cells were selected using 500 µg/ml G418 (Life Technologies, Inc.) in F12FC5. Resistant colonies were isolated and assayed for the presence of SREBP and GFP protein by Western Blotting and by examining GFP green protein by fluorescence microscope with a Nikon Diaphot-200. Colonies expressing SREBP2-GFP were expanded and maintained in F12FC5 containing 500 µg/ml G418.

Calcium Uptake Assay

Kinetic measurements of calcium were performed by a dipping technique as described previously (Yarom *et al.* 1985). Briefly, cells were plated on glass coverslips at a density of 5×10^5 cells/35 mm dish. After an overnight incubation in F12FC5,

coverslips were sequentially washed in a reference buffer (123 mM NaCl, 3.0 mM KCl, 0.4 mM MgCl₂, 0.5mM NaH₂PO₄, 0.25mM Na₂HPO₄, 5.5 mM glucose). At time 0, the coverslips were dipped in a beaker containing Ca²⁺ (1.8 mM) and ⁴⁵Ca²⁺ (4 µCi/ml) in reference buffer with or without 25-hydroxycholesterol (5 µg/ml). After the indicated period of time, cells were washed with calcium free reference buffer and solubilized in 1% SDS, and radioactivity was measured by liquid scintillation.

Transient Transfections with Enhanced Green Fluorescent Protein EGFP-cPLA₂

Cells were seeded on ethanol-washed 22-mm glass coverslips at density of 1 x 10⁵ cells/60 mm plate and transiently transfected with 6 µg of the expression vector encoding a cPLA₂-GFP fusion protein (pEGFP-cPLA₂) or a control vector pEGFP-N3 with the Stratagene Transfection MBS Mammalian Transfection Kit according to the instruction manual (Promega). Cells were incubated for 48 h before a 4-h treatment with 25-OHC (5 µg/ml) and EGFP expression was examining GFP green protein by fluorescence microscope with a Nikon Diaphot-200. Digital deconvolution of the images was performed with Image Pro software (Oncor, Gaithersburg, MD).

Arachidonic Acid (AA) Release

CHO-K1 or OX^R cells, plated at 50,000 cells per dish, were labeled by incubation for 24 h in 1 ml of serum-free medium containing [³H] AA (1 µCi/ml) and 0.1% (W/V) fatty acid-free BSA. After labeling, cells were washed three times with PBS, re-fed with F12FC5, and rested for 1 h. In experiments utilizing metabolic inhibitors (10 or 15 µM AACOCF₃, 20 µM BEL, or 100 µM nifedipine), their addition to the culture medium was at this point. These inhibitor concentrations were determined to produce optimal effects in preliminary experiments. Cells were then refed fresh medium containing either 0.03% ethanol (vehicle control) or 25-OHC (3 µg/ml in ethanol) and the various inhibitors for

the indicated times. Radioactivity in supernatant fractions and cell lysates (in 1% triton X-100) were measured by liquid scintillation counting. The percent release of AA was calculated as the $[\text{Medium dpm}/(\text{Medium dpm} + \text{Cells pm})] \times 100$ and was then normalized to the value of unstimulated controls.

Isolation of Permanent Transfectants Expressing Peroxisome Proliferator Response Element (PPRE) Luciferase Reporter ((AOX)3 TK-Luc)

CHO-K1 cells were grown in Ham's F-12 medium containing 5% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (F12FC5) at 37 °C and 5% CO₂ and co-transfected with 6 µg of the expression vector encoding a peroxisome proliferator response element (PPRE) luciferase reporter ((AOX)3 TK-Luc) and 2 µg of selected marker pWL-NEO by the lipofectamine method using a mammalian transfection kit (Stratagene, La Jolla, CA). The neomycin-resistant cells were selected using 500 µg/ml G418 (Life Technologies, Inc.) in F12FC5. Resistant colonies were isolated and assayed for the presence of (AOX)3 TK-Luc by luciferase activity using the Promega firefly luciferase reporter assay kit according to the instruction manual. Colonies expressing (AOX)3 TK-Luc were expanded and maintained in F12FC5 containing 500 µg/ml G418. Stably transfected cells were then treated with 5 µM 15d-PGJ₂ and 5 µg/ml 25-OHC for 24 h. Normalized luciferase activity were determined and plotted as fold activation relative to untreated cells.

Isolation of Eicosanoids with Sep-Pak C18 Cartridges

CHO-K1 cells, plated at 1×10^6 cells per dish, were labeled by incubation for 24 h in 10 ml of serum-free medium containing [³H] AA (1 µCi/ml) and 0.1% (W/V) fatty acid-free BSA. After labeling, cells were washed three times with PBS, re-fed with F12FC5, and rested for 1 h. Cells were then re-fed fresh medium containing either 0.03% ethanol

(vehicle control) or 25-OHC (3 µg/ml in ethanol) for the indicated time. Eicosanoids were isolated essentially as described by Ma *et al.* (1998). Briefly, medium and cells were harvested by scraping with a rubber policeman. The reaction was terminated by adding 3 ml of ethanol, and the mixture was kept at room temperature for 5 min. Then, 7 ml of water was added to adjust the final concentration of ethanol to 15%. The mixture was centrifuged at 500g for 10 min, and the supernatant was further acidified to pH 3.0 with 1 N HCl. Sep-Pak vac RC C₁₈ cartridges (Waters Associates) were used to isolate eicosanoids from the incubation mixtures. The Sep-Pak cartridges were conditioned before loading the samples. To remove non-eicosanoid bound lipids and other contaminants, the cartridge was washed sequentially with 5 ml of 15% ethanol, 20 ml water and 6 ml petroleum ether. The fractions of eicosanoids were eluted with ethyl acetate and the solvent was evaporated under nitrogen. The dried material was dissolved in ethanol for HPLC analysis.

Measurement of Arachidonate Metabolites by Reverse Phase High-Performance Liquid Chromatography

Extracted [³H] AA metabolites were identified using reverse phase (RP) high-performance liquid chromatography (HPLC). HPLC was performed on a 10-µm Versapack RP C-18 column (300 × 4.1 mm; Alltech Associates, Inc.) using a Beckman 110B liquid chromatography system. The elution program was a 15-min linear gradient of 30% acetonitrile in water, a 5-min linear gradient to 50% acetonitrile, and a 25-min isocratic elution at that acetonitrile concentration. This was followed by a 5-min linear gradient to 73% acetonitrile and a final isocratic elution. All solvents contained 0.1% acetic acid. The flow rate was 0.5 ml/min, and the eluate was detected by Radioactive Flow detector, Flo-one/Beta (Radiomatic Instruments).

Measurement of Caspase-3, -8, and -9 Protease Activity

CHO-K1 cells were plated and treated without and with 25-hydroxycholesterol for different periods of time. After treatment, both treated and nontreated cells were harvested and collected by centrifugation (800g for 10 min). Cells were washed twice with ice-cold PBS and lysed with cell lysis buffer (10 M Tris (pH 7.5), 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaPi, and 10 mM NaPPi). Samples were incubated on ice for 10 min, passed through a 21-gauge syringe 15-times, and then centrifuged at 15,000g for 20 min at 4 °C. Protein concentration in the supernatant (total cell lysate) was measured by the micro-BCA kit. Triplicates of 250 µg of total cell lysate protein were incubated with 20 µM of caspase-3 substrate Ac-DEVD-AMC, caspase-8 substrate Ac-IETD-AFC, or caspase-9 substrate Ac-LEHD-AFC for 1.5 h at 37 °C in protease assay buffer (20 mM Hepes, pH 7.5, 10 % glycerol, 2 mM dithiothreitol). Liberated AMC from Ac-DEVD-AMC was measured on a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 440 nm and liberated AFC from Ac-IETD-AFC and Ac-LEHD-AFC was measured on a spectrofluorometer with an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Immunoblot Analysis of Cytochrome C and Poly (ADP-ribose) Polymerase (PARP)

On day 0, cells were seeded at 2×10^6 /100-mm culture dish in F12FC5. On day 1, the medium was removed, and the cells were rinsed twice with PBS. The cells were then incubated for 24 h in F12FC5 in the presence of 0.03% EtOH or 3 µg/ml 25-OHC. Nuclear extracts, mitochondria, and cytosol fractions were prepared essentially as described by Hua *et al.* (1996). Briefly, cells (2×10^6) were harvested by centrifugation at 600g for 10 min at 4 °C. The pellets were washed once with ice-cold PBS and resuspended with 1 ml of ice-cold buffer C (10 mM Hepes-KOH at pH 7.4, 0.42 M NaCl, 2.5 % (v/v) glycerol, 1.5 mM MgCl₂, 0.5 mM sodium EDTA, 0.5 mM EGTA, 1mM

dithiothreitol) and a protease inhibitor mix (PIM) (Hua *et al.* 1996). The cell suspension was homogenized, on ice, by passage 15 times through a 22-gauge needle. The homogenates were centrifuged twice at 750 x g for 10 min at 4 °C to remove nuclei. Poly(ADP-ribose) polymerase, as well as proteolytic fragments derived from it, were extracted from the nuclear pellet with 100 µl of ice-cold buffer D [10 mM Hepes at pH 7.6, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM EGTA, 0.5 mM dithiothreitol and PIM] for 30 min at 4 °C and cleared of debris by a 20 min spin at the top speed of an Eppendorf microcentrifuge.

The post-nuclear supernatant fraction were centrifuged at 10,000 x g spin for 15 min at 4 °C, and the resulting mitochondria-enriched pellets were resuspended in 100 µl buffer C + PIM (cold). The post-mitochondrial supernatant was further centrifuged at 100,000 x g for 1 h at 4 °C to remove membrane contaminants and the resulting supernatant was used for cytosolic protein determinations. Protein concentration was determined using a micro-BCA kit (Pierce). Samples of the nuclear extracts, mitochondria, and cytosol fractions were mixed with 2× SDS loading buffer (Bollag and Edelstein 1991). Nuclear extracts samples were subjected to SDS-polyacrylamide gel electrophoresis on 7.5 % gels, which was used to detect PARP and the samples of mitochondria and cytosolic fraction were analyzed on 12 % gels to determine cytochrome C. Following electrophoresis, the proteins were transferred to Immobilon-P transfer membrane (Miliopore). The blots were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1 % Tween 20 (TBS-T) followed by a 1 h incubation with either anti-PARP and anti-cytochrome C in TBS-T. The blots were then rinsed 5 times with TBS-T and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:100,000 in TBS-T. The blots were rinsed 5 times with TBS-T, and ECL was performed using SuperSignal Chemiluminescent Substrate kit according to the manufacturer's instructions (Pierce).

CHAPTER 3

RESULTS

25-Hydroxycholesterol Induces Apoptosis in CHO-K1 Cells- a Model for the Cytotoxic Effect of Oxidized LDL

Apoptosis, a form of genetically programmed cell death, plays a key role in regulation of cellularity of the arterial wall. During atherogenesis, deregulated apoptosis may cause abnormalities in arterial morphogenesis, in wall structural stability, and in metabolisms (Bennett *et al.* 1995; Geng and Libby 1995; Han *et al.* 1995; Kockx 1998). Many biophysiologic and biochemical factors, including mechanical forces, reactive oxygen and nitrogen species, cytokines, growth factors, oxidized LDL, etc. may influence apoptosis of vascular cells (Wertz and Hanley 1996; Escargueil-Blanc *et al.* 1997). Among these inducers, oxLDL is believed to play a key role as a triggering molecule that causes injury to the endothelium as an early event in atherosclerosis (Dimmeler *et al.* 1997). OxLDL induces morphological changes and DNA fragmentation characteristic of apoptosis in cultured smooth muscle cells, macrophages, endothelial cells, and lymphoid cells (Orrenius *et al.* 1989; Dimmeler *et al.* 1997; Escargueil-Blanc *et al.* 1998; Arends *et al.* 1990).

Cholesterol oxides or oxysterols have been demonstrated to exist both in oxLDL and in atherosclerotic plaque (Brown and Jessup 1999). The cytotoxicity of oxysterols to many cell types has also widely been reported including vascular cells such as endothelial cells (Ramasamy *et al.* 1992), macrophages (Aupeix *et al.* 1995; Clare *et al.* 1995), smooth muscle cells (Peng *et al.* 1979; Hughes *et al.* 1994), and lymphocytes (Christ *et al.* 1993). Therefore, it is likely that oxysterols present in oxLDL, at least in part, are causative agents of the apoptotic cell death that occurs in atherosclerotic

lesion. However, it is not clear how oxLDL or its active components, oxysterols, induce apoptosis in these and other vascular cells. In order to investigate the mechanism by which oxLDL induced apoptosis, we first tried to create a simple model system. CHO cells are well suited for mutational analysis of complex cellular pathways (Leonard and Sinensky 1988). It is possible to use transfected or mutant CHO cell lines for genetic analysis of the gene products and functions involved in the programmed cell death induced by oxLDL or its components. In this part of the study, we used CHO-K1 cells transfected with CD36 to study the cytotoxic effects of oxLDL and a model oxysterol, 25-hydroxycholesterol. Because there is no direct evidence that oxysterols contribute to the cytotoxicity of oxLDL, we also isolated a CHO/CD36 mutant cell line resistant to killing by 25-hydroxycholesterol and then tested whether this mutant is cross resistant to killing by oxLDL in order to verify the hypothesis that the induction of apoptosis by oxLDL is mediated by oxysterols.

Transfection of CD36 into CHO-K1 Cells Confers the Ability to Take Up OxLDL

Chemical modifications of LDL, such as oxidation, convert LDL into a high affinity ligand of scavenger receptors. OxLDL is cytotoxic for many cell types in the vessel wall, and its interaction with the macrophage scavenger receptors and subsequent toxic effects play a crucial role in the initiation of the atherosclerotic lesion. To test if uptake of oxLDL is sufficient to elicit cytotoxic effects in a fibroblastic cell line, we have transfected CHO-K1 with CD36, a scavenger receptor structurally related to SR-BI and its human counterpart CLA-I. This receptor has been reported to bind oxLDL and acetylated LDL. (Endemann *et al.* 1993; Calvo *et al.* 1998). Cell association of oxLDL to CHO-K1 expressing CD36 was examined by fluorescence microscopy after incubating Dil-labeled oxLDL with CHO-K1 stably transfected with a vector carrying CD36FLAG (CHO/CD36). Untransfected CHO cells displayed a diffuse light staining, possibly due to

traces of free Dil or uptake through other scavenger receptors (Figure 1A). However, when CHO/CD36 cells were incubated with Dil-oxLDL, a subset of cells, corresponding to the number of cells expressing CD36 showed both an internal punctuated staining pattern (probably endosomes and lysosomes) and an intense plasma membrane staining (Figure 1B). These findings confirm previous reports (Nicholson *et al.* 1995) that CD36 when expressed on CHO cells binds and allows internalization of oxLDL

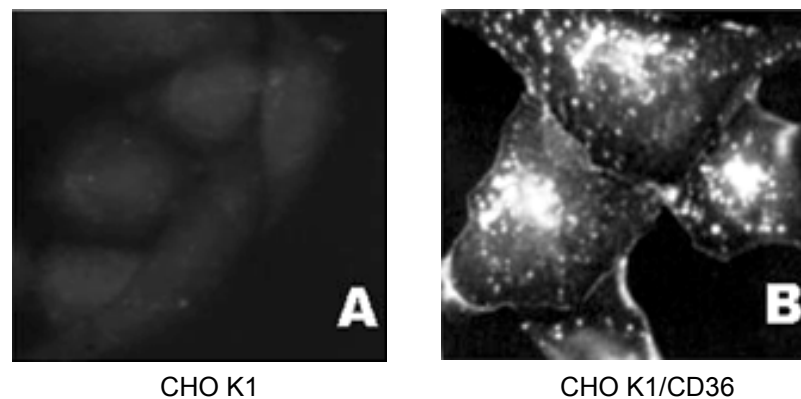


Figure 1. Binding and Uptake of Dil-Labeled OxLDL by CD36-Expressing CHO-K1 Cells. CHO-K1 cells were transfected with the vector pcDNA3 carrying the human CD36FLAG cDNA (CHO/CD36). Transfected cells were grown on glass coverslips and then incubated with 10 μ g/ml Dil-oxLDL for 1 h at 37 °C. After washing with PBS, cells were fixed in 4% formaldehyde in PBS and mounted. Cell association of Dil-labeled oxLDL was observed under fluorescence microscopy. A, CHO-K1 cells; B, CHO/CD36 cells.

OxLDL is Cytotoxic to CHO/CD36

Using a single-cell-plating assay to determine cytotoxicity, we found that expression of CD36 in CHO cells renders the cells susceptible to killing by oxLDL (Figure 2). In this assay, 500 cells are plated on 35-mm dishes, subjected to different treatments, and allowed to form colonies, which can be stained and counted. Figure 2

shows that the treatment of CHO/CD36 with 10 μ g/ml of oxLDL for 5 days eliminates formation of colonies, indicating cytotoxicity, whereas untransfected CHO-K1 cells were not affected by this treatment.

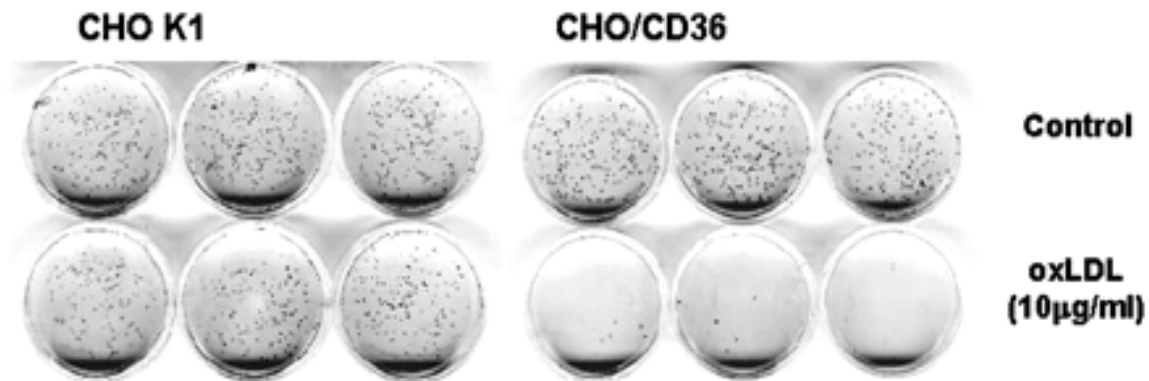


Figure 2. Effect of OxLDL on CHO-K1 and CHO/CD36 Cells. Cells were seeded at 500 cells/35-mm culture dish in F12FC5 and incubated overnight. The culture medium was then changed to F12FC5 containing 10 μ g/ml oxLDL or F12FC5 (control). The cells were incubated 5 days and fixed, and colonies were stained as described in Materials and Methods.

OxLDL Induces Apoptosis in CHO/CD36

OxLDL induced apoptosis in a dose-dependent manner (Figure 3). Approximately 10 or 20% of the CHO/CD36 cells became TUNEL-positive after a 48 h incubation with 10 or 50 μ g of protein/mL of oxLDL, respectively (Figure 3A). Commensurate with the results derived from the TUNEL assay, DNA ladders were present in genomic DNA extracted from CHO/CD36 cells incubated with similar oxLDL concentrations (Figure 3B). The fragmented DNA showed the distinct pattern of oligonucleosomes found in apoptotic cells. These findings indicate that oxLDL is able to induce apoptosis in cultured fibroblasts when the cells are able to bind and/or internalize oxLDL.

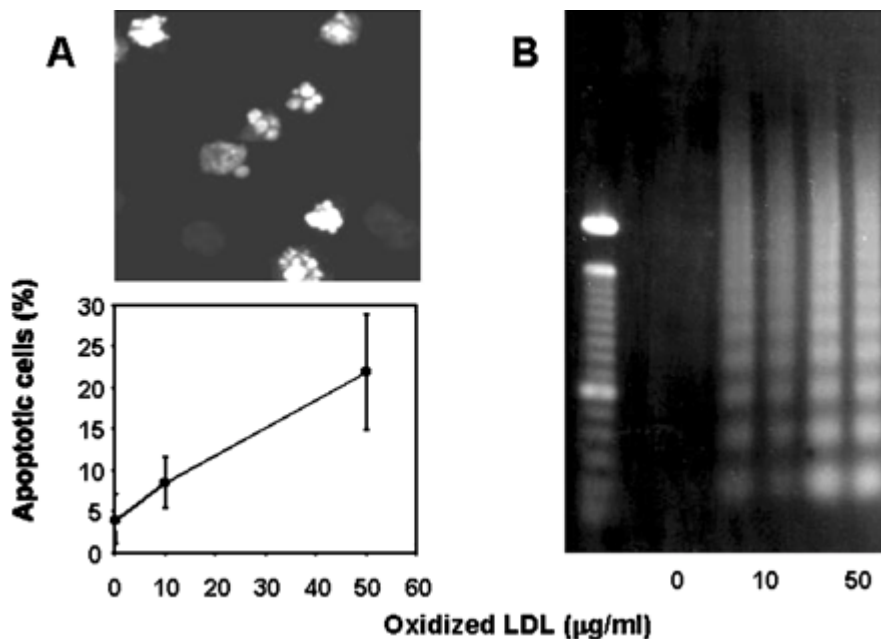


Figure 3. OxLDL Induces Apoptosis in CHO/CD36 Cells. A, cells (50,000) were plated on glass coverslips and incubated for 48 h in F12FC5 containing 0, 10, or 50 μg/ml oxLDL. After washing with PBS, the cells were fixed, and apoptosis was evaluated by an *in situ* TUNEL reaction as described under "Experimental Procedures." The graph represents the average percentage of TUNEL-positive cells in 10 40 × fields ± SD. B, cells at an initial density of 5×10^5 were cultured for 24 h in F12FC5, with 0, 10, or 50 μg/ml oxLDL, and then harvested, lysed, and fragmented DNA was detected on agarose gels as described in Materials and Methods.

25-Hydroxycholesterol Induces Apoptosis in CHO-K1 Cells

OxLDL is a chemically complex particle. Which component is responsible for its cytotoxicity? Several recent studies have shown that much of the cytotoxicity of oxLDL is associated with the neutral lipid components, in particular the oxysterols (Chisolm *et al.* 1994; Sevanian *et al.* 1995). In order to test whether oxysterol is cytotoxic to the cells, a model oxysterol compound, 25-hydroxycholesterol was used. Oxysterols inhibit cholesterol synthesis. In the serum free medium, this is the reason for cytotoxicity. In

order to exclude this possibility in our studies, we treated cells with 25-hydroxycholesterol in the presence of serum as the exogenous source of cholesterol.

Figure 4 shows the results of a representative single-cell plating experiment as a function of time of exposure to 3 $\mu\text{g/ml}$ 25-hydroxycholesterol in the presence of serum. A significant reduction (~50%) in the number of viable cells was seen after a 6 h incubation. Furthermore, after a 12 h incubation, virtually all treated cells were dead. The mode of cell death was confirmed to be through apoptosis by TUNEL assay (Figure 5A) and oligonucleosomal DNA laddering assay (Figure 5B).

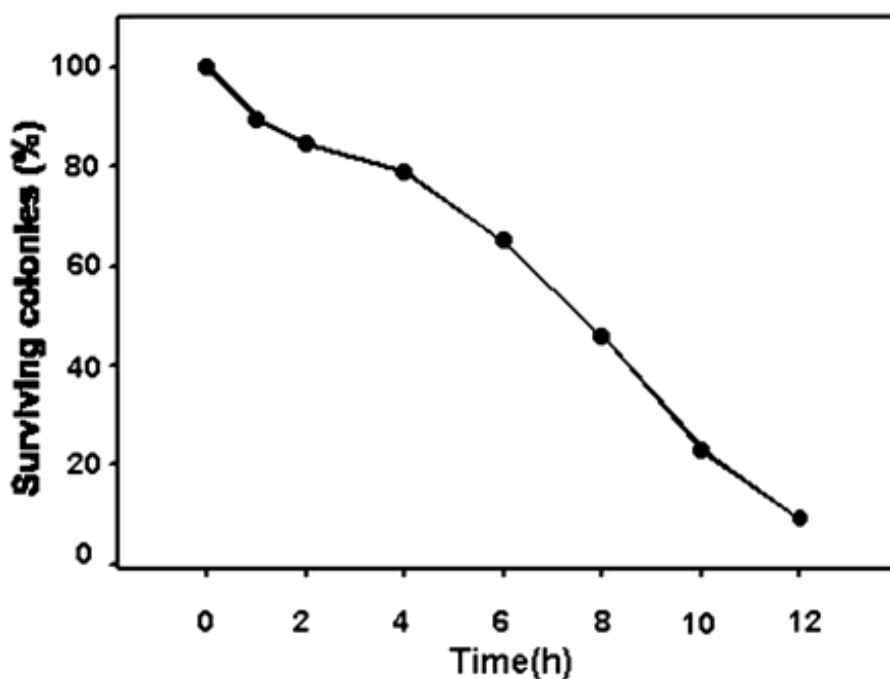


Figure 4. Time Course Killing of CHO-K1 Cells by 25-Hydroxycholesterol. CHO-K1 cells were seeded at 500 cells/35-mm culture dish in F12FC5 and incubated overnight. The culture medium was then changed to F12FC5 supplemented with 0.03% EtOH (control) or 3 $\mu\text{g/ml}$ 25-hydroxycholesterol. Cells were then incubated for 0, 1, 2, 4, 6, 8, 10, or 12 h. After each time point, the medium was removed, the cells were rinsed and refed F12FC5 for 5 days, and then surviving colonies were fixed, stained, and counted.

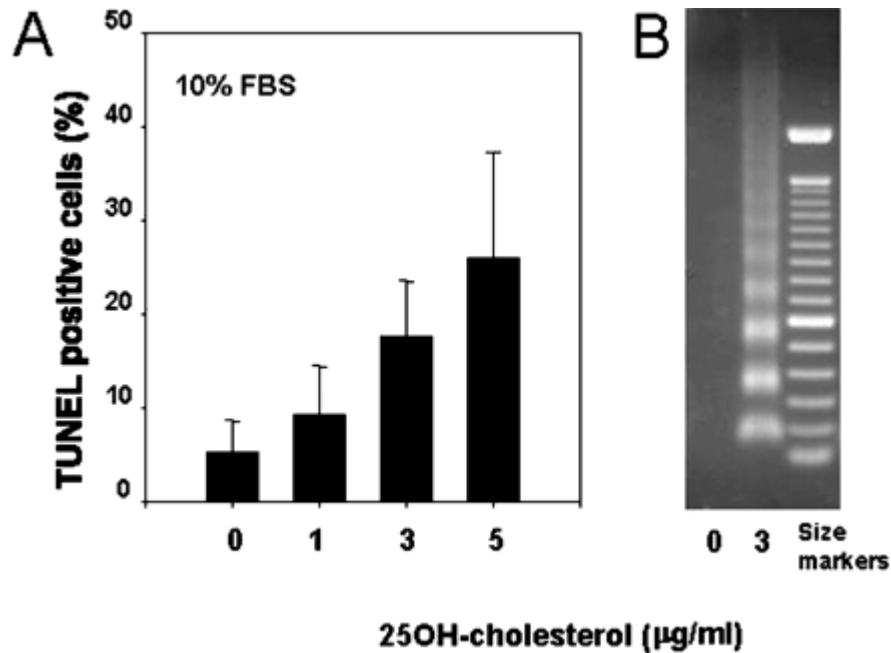


Figure 5. 25-Hydroxylcholesterol Induces Apoptosis in CHO/CD36 Cells. *A*, apoptosis assay. Cells (30,000) were plated on glass coverslips and incubated for 24 h in F12FC5 containing 0, 1, 3, or 5 µg/ml 25-hydroxycholesterol. Apoptosis was measured by TUNEL as described in Materials and Methods. *B*, gel electrophoresis of DNA. Cells at an initial density 5×10^5 were cultured for 24 h in F12FC5, with or without 3 µg/ml 25-hydroxycholesterol, and incubated for 24 h and then harvested and lysed, and DNA fragments were detected as described in Materials and Methods.

The 25-Hydroxycholesterol-Resistant Mutant Cell Line (OX^R) is Cross-Resistant to OxLDL

If oxysterols are the mediators of the cytotoxic effects of oxLDL, an oxysterol-resistant mutant should be resistant to killing by oxLDL. We mutagenized CHO/CD36 with methanesulfonic acid ethyl ester and selected colonies that grew in medium containing 3 µg/ml 25-hydroxycholesterol (25-OHC). As shown in Figure 6A, OX^R cells were resistant to killing by 3 µg/ml 25-hydroxycholesterol. These cells were also resistant to killing by 35µg/ml oxLDL (Figure 6B). Figure 6C shows that the staining pattern of OX^R cells with Dil-oxLDL is similar to that in CHO/CD36, demonstrating that binding and internalization of oxLDL in the OX^R cell line is unaltered from its parental cell line. A general defect in one or more steps in the apoptosis pathway *per se* (e.g. defective caspase 3, overexpression of Bcl-2, etc.) would produce a similar resistance phenotype. Therefore, we also confirmed that OX^R cells were capable of undergoing apoptosis in response to another known apoptosis inducer. Figure 6A shows that OX^R cells are susceptible to killing by staurosporine, a reagent that induces apoptosis through inhibition of protein kinase C, and therefore seemed to have a functioning apoptosis pathway, at least in the execution phase of the death program.

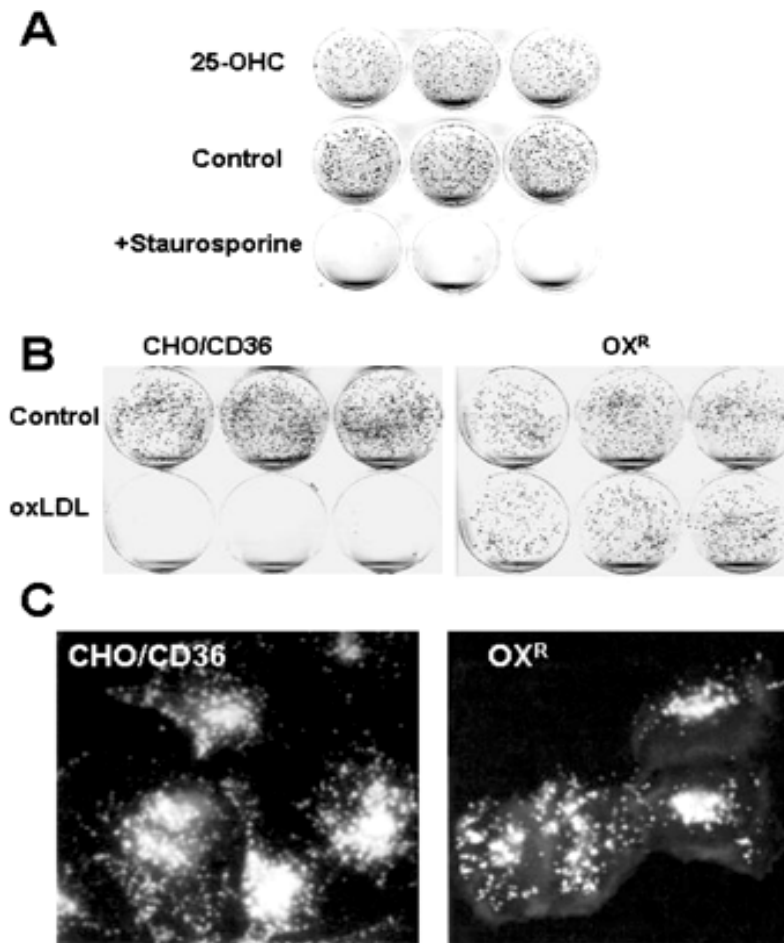


Figure 6. Isolation of a 25-Hydroxycholesterol-Resistant CHO/CD36 Mutant. CHO-K1 cells were mutagenized by treatment with methanesulfonic acid ethyl ester. Mutants were selected from the colonies that survived in 3 $\mu\text{g/ml}$ 25-OHC. **A**, OX^R cells were seeded at 500 cells/35-mm culture dish in F12FC5 medium and incubated overnight. The medium was then changed to F12FC5 and 0.03% EtOH (control), 3 $\mu\text{g/ml}$ 25-OHC, or 1 μM staurosporine, as indicated to the left. After 24 h, the medium was removed, and the cells were rinsed and refed F12FC5 for 5 days and then fixed and stained. **B**, OX^R cells were plated in F12FC5 at a density of 1000 cells/60-mm plate. On day 2, cells were treated with or without 10 $\mu\text{g/ml}$ oxLDL for 6 days; cells were then washed with PBS, and the medium was changed back to F12FC5. Colonies were allowed to grow for another 2 days and then fixed and stained. **C**, CHO/CD36 and OX^R cells were grown on glass coverslips and then incubated with 10 $\mu\text{g/ml}$ Dil-oxLDL for 1 h at 37 °C. After washing with PBS, cells were fixed in 4% formaldehyde in PBS and mounted. Cell association of Dil-labeled oxLDL was observed by fluorescence microscopy.

Cytotoxic Mechanism of Apoptosis Induced by 25-Hydroxycholesterol in CHO-K1 Cells

The above results have shown that expression of a receptor for oxLDL (CD36) in CHO-K1 cells resulted in a cell line capable of extensive uptake of oxLDL from culture medium. In contrast to the parental cells, these transformants became sensitive to the induction of apoptosis by low levels (10-50 µg/mL) of oxLDL. CHO-K1 cells were also shown to be sensitive to the induction of apoptosis by the model oxysterol, 25-OHC, and a somatic cell mutant isolated as 25-OHC resistant was cross resistant to killing by oxLDL. These studies supported the proposition that oxysterols are among the pro-apoptotic components of oxLDL. Thus we can determine apoptotic pathways using a simple model oxysterol, 25-OHC as an inducer in the highly genetically manipulatable cell line: CHO-K1.

Oxysterol-Induced Apoptosis Does Not Involve Transcriptional Control of Cholesterol Biosynthesis

The best-known biological activity of oxysterols is transcriptional repression of cholesterol biosynthesis, through inhibition of processing of the sterol regulatory element binding proteins (SREBPs), although other activities for these molecules have recently come to be appreciated (Edwards and Ericsson 1999). Oxysterols have been reported to initiate apoptosis in CEM leukemic cells by interfering with the synthesis of cholesterol in the absence of an exogenous cholesterol source (Ayala-Torres *et al.* 1997). Because we carried out our incubations in the presence of serum, as an exogenous cholesterol source, we would expect that the apoptotic pathway would not be mediated by inhibition of cholesterol synthesis. However, we directly examined the possibility that oxysterol killing by apoptosis involved regulation of SREBP proteolysis and the cholesterol biosynthetic pathway.

Somatic cell mutants of CHO cells, resistant to inhibition of growth by 25-OHC in the absence of an exogenous cholesterol source, have been extremely useful in defining the role of SREBPs in the regulation of cholesterol biosynthesis (Chang *et al.* 1997; Leonard and Sinensky 1988). There are 2 classes of oxysterol-resistant mutants characterized to date: class 1 mutants, which constitutively produce a truncated SREBP-2 that acts as a mature transcription factor, and class 2 mutants, which are defective in SREBP cleavage-activating protein, which appears to transduce the oxysterol-mediated inhibition of SREBP proteolysis. Two lines of evidence using some of these mutants suggest that SREBP proteolysis regulation is not involved in oxysterol-induced apoptosis. First, CHO-K1 cells transfected with constitutively active SREBP-2 are resistant to inhibition of growth by $<0.3 \mu\text{g/ml}$ 25-hydroxycholesterol in sterol-free medium (Figure 7A) but are still killed by $3 \mu\text{g/ml}$ 25-hydroxycholesterol in medium supplemented with serum (Figure 7B). Second, we also compared OX^R cells to CR1 cells, a 25-hydroxycholesterol-resistant mutant isolated in cholesterol-free medium (Sinensky *et al.* 1979), which has been recently found to be a class 2 mutant by our laboratory colleagues. This line shows constitutive expression of both SREBP1 and SREBP2 in cholesterol-free medium supplemented with 25-hydroxycholesterol as reported by our laboratory (Rusinol *et al.* 2000) but is still sensitive to killing by $3 \mu\text{g/ml}$ 25-hydroxycholesterol in medium supplemented with serum (Figure 8).

The resistance of CR1 to inhibition of growth in cholesterol-free medium (Figure 8) is at a concentration of 25-hydroxycholesterol ($0.2 \mu\text{g/ml}$), where killing of wild-type cells does not occur in medium supplemented with cholesterol (Sinensky *et al.* 1979). Resistance of CR1 to killing, under these conditions, reflects the constitutive processing of SREBPs and the resultant loss of transcriptional control of cholesterol biosynthesis

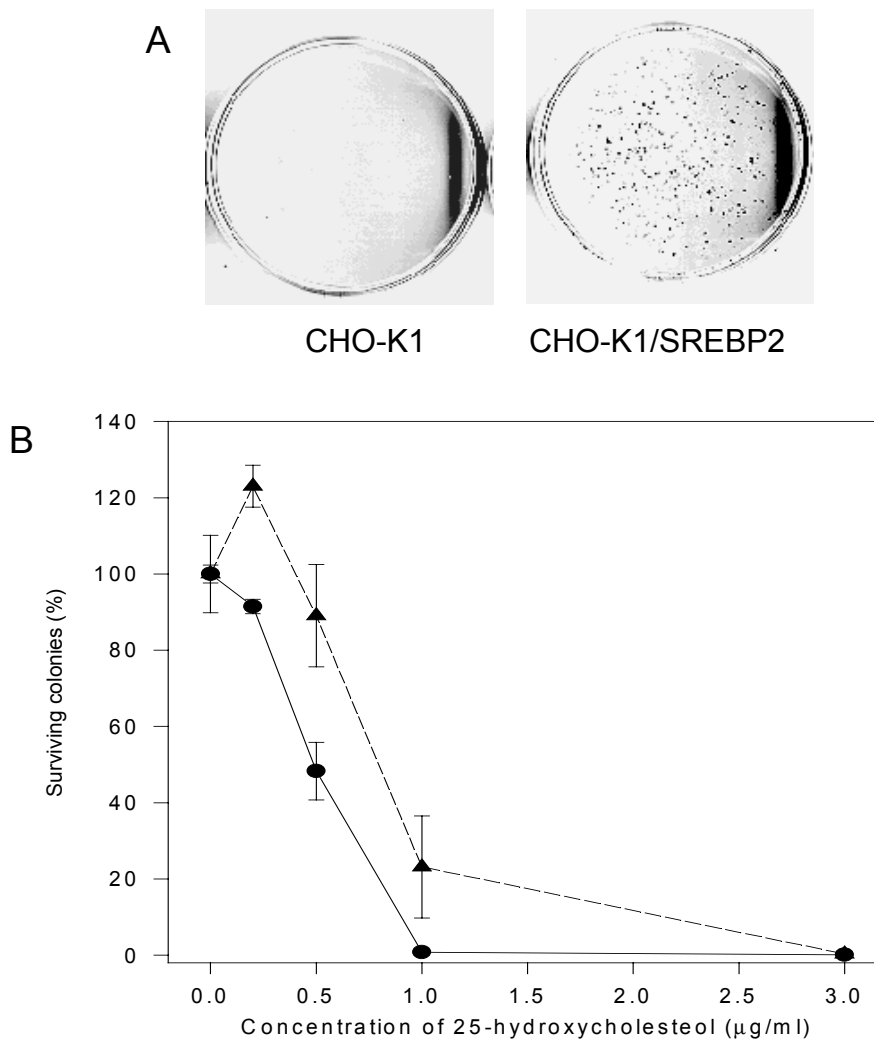


Figure 7. Effects of 25-Hydroxycholesterol on the Killing of CHO-K1 Cells and CHO-K1 /SREBP2 Transfected Cells in the Absence and Presence of Serum. **A**, CHO-K1 and CHO-K1 /SREBP2 cells were seeded at a density of 1000 cells per 60-mm plate and permitted to attach in F12FC5 overnight. The medium was then switched to F-12 plus 1% Nutridoma (cholesterol-free medium) and 0.2 μg/ml 25-hydroxycholesterol in F-12 plus 1% Nutridoma, incubated for another 5 days, and then fixed and stained. **B**, CHO-K1 cells (●) and CHO-K1 /SREBP2 transfected cells (▲) were seeded at 500 cells/35-mm culture dish in F12FC5 and incubated overnight. The culture medium was then changed to F12FC5 supplemented with 0.03 % EtOH (control) or 3 μg/ml 25-hydroxycholesterol. Cells were then incubated for 24 h. The medium was removed, the cells were rinsed and refed F12FC5 for 5 days, and then surviving colonies were fixed, stained, and counted.

(Peffley *et al.* 1988), mediated by SRE1 elements. Sensitivity of OX^R to 25-hydroxycholesterol under these conditions suggests that cholesterol biosynthesis is still transcriptionally controlled in this mutant. This hypothesis was tested directly in our laboratory by means of an SRE1 reporter construct, pTK(K×3)CAT (Dawson *et al.* 1988), for transcriptional control by sterols. Comparison of the response of this reporter to treatment with 25-hydroxycholesterol in CR1 and OX^R (Rusinol *et al.* 2000) indicates that regulation of SRE1 in OX^R cells is normal in these cells, in contrast to the loss of transcriptional control in CR1.

This result also suggests that uptake and intracellular transport of 25-hydroxycholesterol is unaffected in OX^R. We checked this directly by determining the incorporation of 25-[³H]hydroxycholesterol into ester in OX^R cells as compared with its parental cell line, CHO/CD36. The results from our lab colleague indicated similar levels of incorporation of label into ester in the two cell lines (Rusinol *et al.* 2000), confirming that uptake and transport of 25-hydroxycholesterol to the endoplasmic reticulum is not affected in the mutant. It might be expected that there would be no role for transcriptional control of cholesterol biosynthesis in the induction of apoptosis by higher concentrations of 25-hydroxycholesterol in medium supplemented with serum and, therefore, an exogenous source of cholesterol. This expectation is confirmed by sensitivity of the transcriptionally constitutive CR1 cells to 3 µg/ml 25-hydroxycholesterol in medium supplemented with serum, in contrast to the resistance of OX^R (Figure 8) under these conditions.

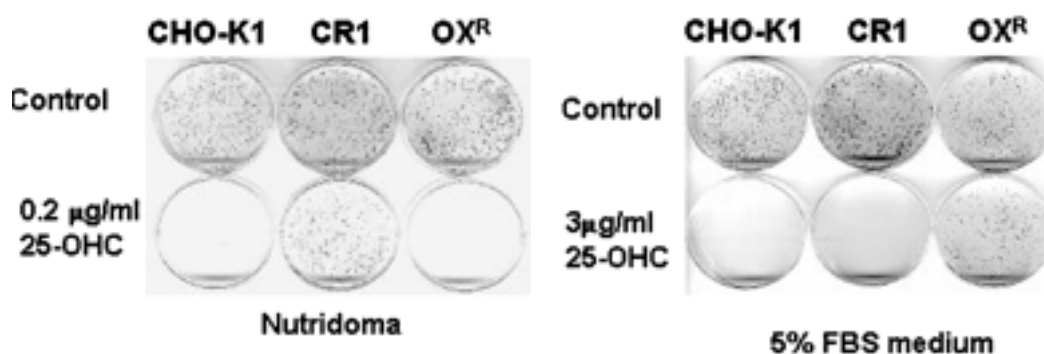


Figure 8. Comparison of the Phenotype of a Cholesterol Regulatory Mutant, CR1, and OX^R. CHO-K1, OX^R, and CR1 cells were seeded at a density of 1000 cells per 60-mm plate and permitted to attach in F12FC5 overnight. The medium was then switched to F-12 plus 1% Nutridoma (cholesterol-free medium) and 0.2 μg/ml 25-hydroxycholesterol or 3 μg/ml 25-hydroxycholesterol in F12FC5, incubated for another 5 days, and then fixed and stained.

Calcium Plays a Critical Role in 25-Hydroxycholesterol Induced Apoptosis

High intracellular calcium levels induced apoptosis in several experimental models (Juntti-Berggren *et al.* 1993; Jiang *et al.* 1994; Ares *et al.* 1997; Ayala-Torres *et al.* 1997) including in response to oxLDL (Escargueil-Blanc *et al.* 1997). 25-Hydroxycholesterol increased the cellular uptake of calcium in a variety of cell types (Boissonneault and Heiniger 1985; Ares *et al.* 1997). Therefore, we investigated the possible involvement of calcium in the initial signaling events in the oxysterol-induced apoptosis. Figure 9A shows that CHO-K1 cells were protected from the killing effects of 3 μg/ml 25-hydroxycholesterol when the treatment was carried out in calcium-free medium. Therefore, in order to confirm that oxysterol affects Ca²⁺ transport, we measured calcium uptake using ⁴⁵Ca²⁺. Figure 9B shows that 3 μg/ml 25-hydroxycholesterol stimulates Ca²⁺ uptake in CHO-K1 with an increase of 3-fold in the first 10 min. Furthermore, when the L-type calcium channel blocker nifedipine was included during the incubations, the 25-hydroxycholesterol-induced cytotoxicity (Figure

9A) and the calcium uptake (Figure 9B) were blocked. Measurement of 25-hydroxycholesterol stimulation of Ca^{2+} uptake in OX^{R} indicated that the mutant was unresponsive (Figure 9C), an observation highly consistent with oxysterol-induced apoptosis being mediated by intracellular Ca^{2+} .

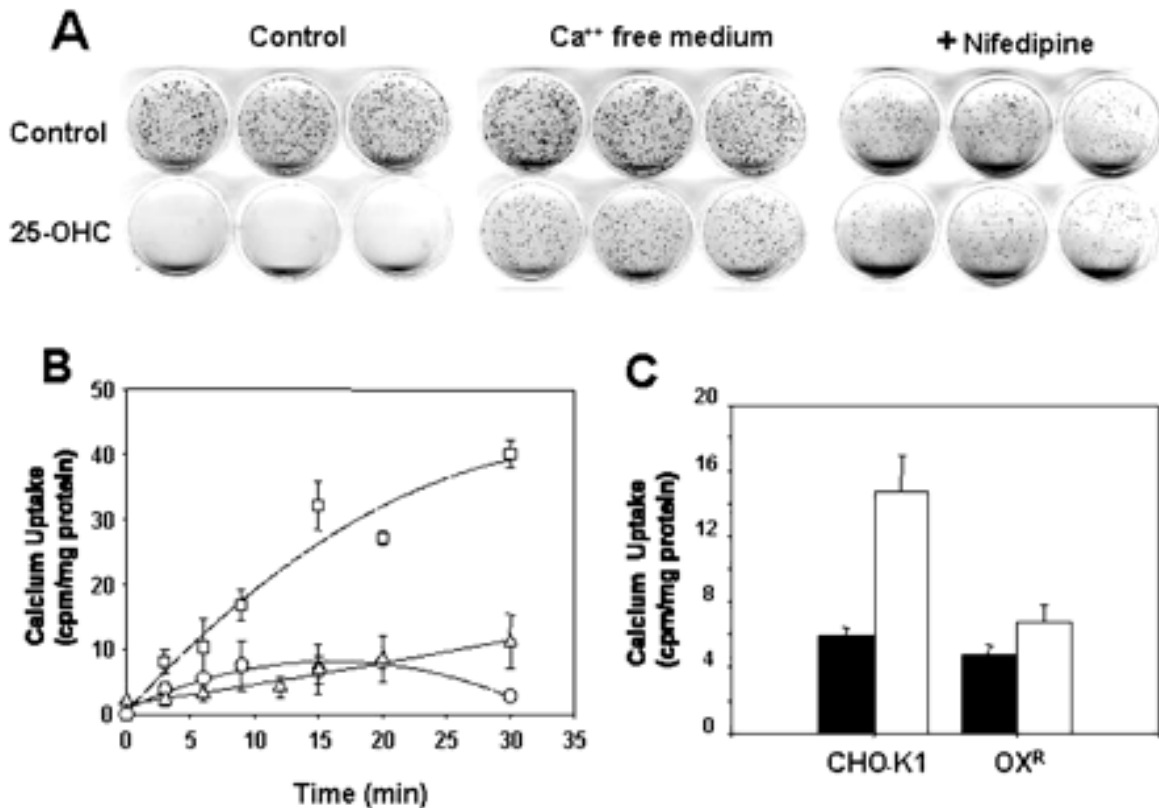


Figure 9. Role of Ca^{2+} Uptake in the Apoptosis Induced by 25-Hydroxycholesterol. A, CHO-K1 cells were seeded at 500 cells/35-mm culture dish in F12FC5 and incubated overnight. The culture medium was then changed to Dulbecco's modified Eagle's medium containing 5% fetal bovine serum medium, with or without Ca^{2+} , or supplemented with nifedipine (100 μM) and 0.03% EtOH (control) or 3 $\mu\text{g}/\text{ml}$ 25-OHC and incubated for 12 h. The medium was removed, and the cells were rinsed and refed F12FC5 for 5 days before being fixed and stained. B, CHO-K1 cells were plated on glass coverslips at a density of 5×10^5 cells/35-mm dish. After an overnight incubation in F12FC5, $^{45}\text{Ca}^{2+}$ uptake was determined as described in 'Materials and Methods' in the absence (○) or presence (□) of 3 $\mu\text{g}/\text{ml}$ 25-hydroxycholesterol or in medium supplemented with both 3 $\mu\text{g}/\text{ml}$ 25-hydroxycholesterol and 100 μM nifedipine (Δ). C, CHO-K1 or OX^{R} cells were seeded as in B and incubated overnight, and $^{45}\text{Ca}^{2+}$ uptake was determined in the absence (solid bars) or presence (open bars) of 3 $\mu\text{g}/\text{ml}$ 25-hydroxycholesterol.

Activation of cPLA₂ by 25-Hydroxycholesterol

An enhanced calcium flux is produced by treatment with 25-OHC and this increase in calcium flux is required for the induction of apoptosis. Calcium is a 2nd messenger. Increases in cytosolic calcium concentration can entrain several signal transduction pathways. One of the signal transduction pathways distal to sustained increases in intracellular Ca²⁺ proceeds through the activation of the 85 kD cytosolic phospholipase A₂ (cPLA₂) (Wolf and Laster 1999; Duan *et al.* 2001). So we determined whether 25-OHC could activate cPLA₂. The standard ways of assaying cPLA₂ activation is the release of radiolabeled arachidonic acid from cells pre-loaded with radioactive arachidonic acid (AA). We found that, indeed, AA release was significantly stimulated by treatment of [³H] arachidonate labeled CHO-K1 cells with 25-OHC (Figure 10) and was blocked by the cPLA₂ inhibitor arachidonyltrifluoromethyl ketone (AACOCF₃) (Figure 10). Because AACOCF₃ can inhibit both calcium-dependent cPLA₂ and calcium-independent PLA₂ (iPLA₂), the iPLA₂ specific inhibitor-bromoenol lactone (BEL) was also tested. The result showed that AA release was not blocked by BEL (Figure 10). These results are consistent with phospholipase A₂ activation by 25-OHC, particularly cPLA₂.

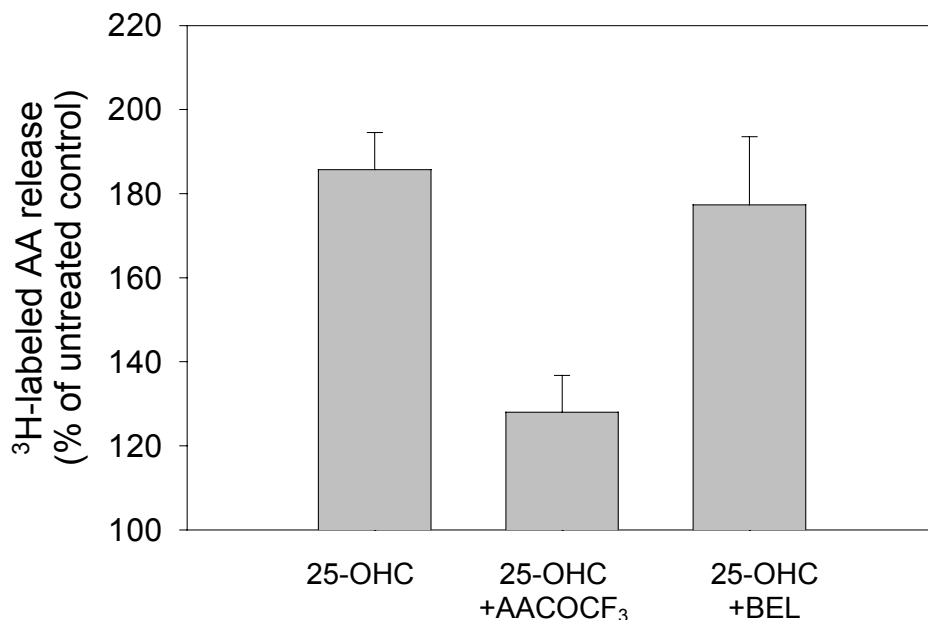


Figure 10. Stimulation of AA Release by 25-OHC in CHO-K1 Cells. CHO-K1 cells were labeled by incubation for 24 h in 1 ml of serum-free medium containing 1 μ Ci/ml [3 H] AA and 0.1% (W/V) fatty acid-free BSA. Post-labeling, cells were washed three times with PBS, re-fed with F12FC5 containing 20 μ M of AACOCF₃ or BEL and rested for 1 h. Cells were then treated with 0.03% EtOH or 3 μ g/ml 25-OHC and 20 μ M of AACOCF₃ or BEL as indicated for 5 h. Radioactivity of supernatants and cell lysates (in 1% triton X-100) were measured by liquid scintillation counting. The amount of the radioactivity released into the supernatant was expressed as a percentage of the total incorporated radioactivity.

To further test the hypothesis that 25-OHC mediates arachidonate release through a calcium-dependent process, we examined the effect of blockage of 25-OHC stimulated calcium uptake on arachidonate release. We have demonstrated that the stimulated calcium uptake by 25-OHC is blocked by nifedipine (100 μ M) in CHO-K1 cells and is constitutively defective in a 25-OHC resistant CHO-K1 cell mutant, OX^R. We, therefore, examined the effect of 25-OHC treatment on arachidonate release in nifedipine treated CHO-K1 cells and in OX^R cells. We found (Figure 11) that in both

models of inhibition of 25-OHC stimulated calcium-uptake, arachidonate release was blocked.

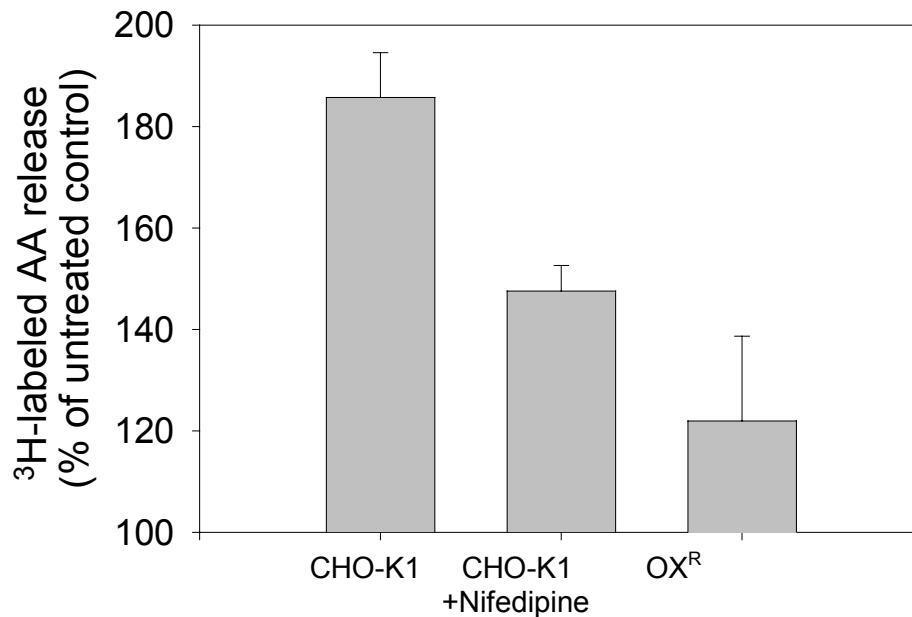


Figure 11. Stimulation of AA Release is Dependent on Calcium Uptake. CHO-K1 or OX^R cells (50,000 cells/dish) were labeled by incubation for 24 h in 1 ml of serum-free medium containing 1 μ Ci/ml [³H] AA and 0.1 % (W/V) fatty acid-free BSA. Post-labeling, cells were washed three time with PBS, re-fed with F12FC5 containing 100 μ M nifedipine, and rested for 1 h. Cells were then treated with or without 3 μ g/ml 25-OHC and 100 μ M nifedipine. Radioactivity of supernatants and cell lysates (in 1 % triton X-100) were measured by liquid scintillation counting. The results are expressed relative to controls not treated with 25-OHC.

The Mechanism of Activation of cPLA₂ by 25-Hydroxycholesterol

We further examined the mechanism of activation of cPLA₂ by 25-OHC. Based on numerous studies, cPLA₂ can be activated by 2 different events: (1) calcium-dependent translocation of the protein to the membrane; (2) phosphorylation *via* classical MAP kinase of Ser 505. In most cell lines studies (Chaminade *et al.* 1999), cPLA₂ is activated by an increase in cellular Ca²⁺ concentration which produces a relocalization of the enzyme from the cytosol to the nuclear and endoplasmic reticulum membranes (Murakami *et al.* 1997, 1998; Chaminade *et al.* 1999). This Ca²⁺-dependent translocation is thought to reflect a translocation that occurs *in vivo* when cells are stimulated with agents that increase intracellular Ca²⁺. To examine whether such relocalization of cPLA₂ occurs in response to the treatment CHO-K1 cells with 25-OHC, indirect immunofluorescence was performed. We used a CHO-K1 cell line that was transiently transfected with the human cPLA₂ as a fusion protein with Green Fluorescent Protein (GFP- cPLA₂) to allow the cell overexpress human cPLA₂. In resting cells, cPLA₂ was found distributed throughout the cytoplasm (Figure 12A). In contrast, after treatment CHO-K1 cells with 5 µg/mL of 25-OHC for 4 hrs, significant cPLA₂ staining appeared as a discrete ring surrounding the nucleus (Figure 12B). A distinct ring was not apparent in untreated cells (Figure 12A), although a low level of perinuclear staining was detectable in some cells. These results show that 25-OHC produces a dramatic relocalization of cPLA₂, consistent with the usual mechanism of Ca²⁺-dependent activation.

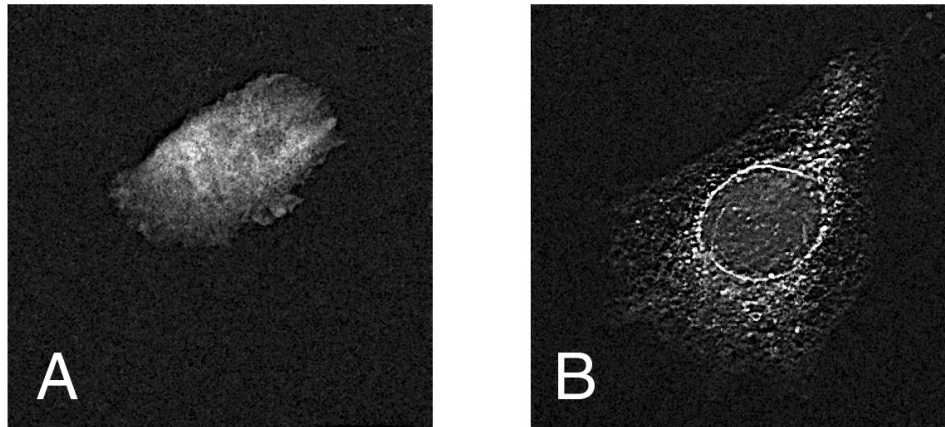


Figure 12. Relocalization of a PEGFP-CPLA₂ Fusion Protein after Treatment of CHO-K1 Cells with 25-OHC. CHO-K1 cells, on coverslips, were examined by fluorescence microscopy and digital deconvolution after transient expression vector of pEGFP-CPLA₂ for 48 h, followed by an addition 4 h incubation in the absence (A) or presence (B) of 25-OHC (5 µg/ml).

The other mechanism by which cPLA₂ is activated is through serine phosphorylation by MAP kinases (Lin *et al.* 1993). Phosphorylation of cPLA₂ produces a slower migration on SDS-PAGE which allows quantitation of the ratio of phosphorylated to non-phosphorylated enzyme by immunoblot. We have performed such experiments in collaboration with Dr. Christina Leslie and have found that, in CHO-K1 cells there is no detectable change in the phosphorylation of Ser 505 in response to 25-OHC (Panini *et al.* 2001).

Activation of CPLA₂ is Involved in 25-OHC Induced Cytotoxicity in CHO-K1 Cells

We have established above that 25-OHC, at concentrations of 3 µg/mL or above, is cytotoxic to CHO-K1 cells in standard culture medium through induction of apoptosis. In order to ascertain whether 25-OHC stimulation of arachidonate release signals the induction of apoptosis, we examined the effect of inhibition of arachidonate release by

AACOCF₃ on 25-OHC induced cytotoxicity in CHO-K1 cells by means of a single-cell plating assay. The results (Figure 13) demonstrate that inhibition of arachidonate release with AACOCF₃ blocks cell killing.

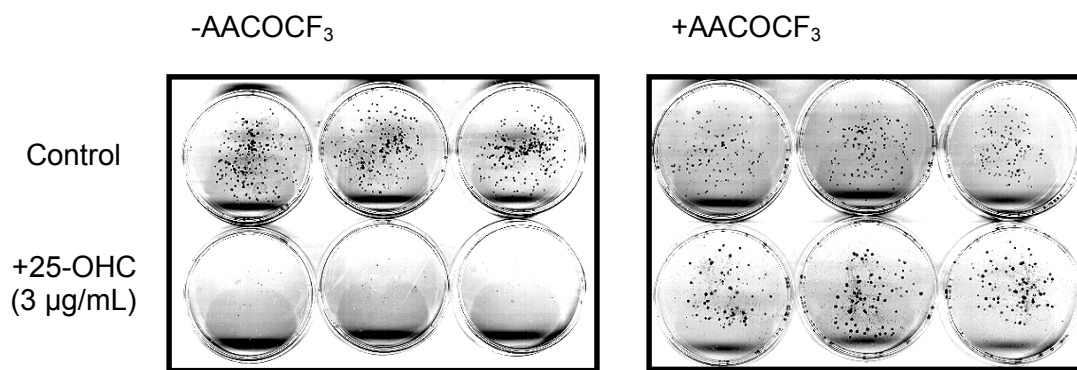


Figure 13. The CPLA₂ Inhibitor AACOCF₃ Blocks the Cytotoxic Effect of 25-OHC. CHO-K1 cells were seeded at 500 cells per 35-mm culture dish in F12FC5 and incubated overnight. The culture medium was then changed to F12FC5 supplemented with AACOCF₃ (10 µM) containing either 0.03% EtOH (control) or 3 µg/ml 25-OHC and incubated for 36 h. The medium was removed, and the cells were washed with PBS and incubated for 5 days in F12FC5 before being fixed and stained.

The arachidonate released may act as a signal through oxidative conversion to other metabolic products. We, therefore, examined the effects of 5,8,11,14-eicosatetraynoic acid (ETYA), an inhibitor of arachidonate metabolism. Figure 14 demonstrates that ETYA could block the killing induced by 25-OHC in agreement with the hypothesis that activation of arachidonate metabolism is involved in the signaling of the induction of apoptosis by 25-OHC.

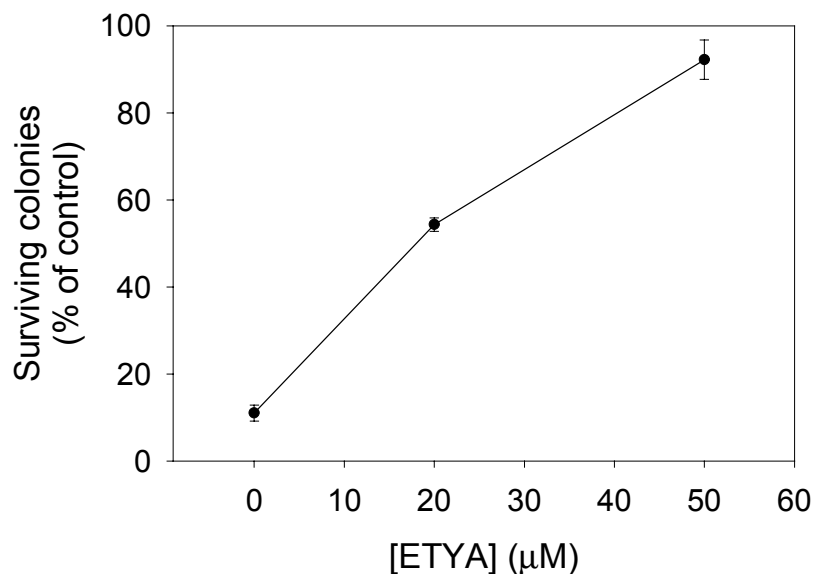


Figure 14. The Killing of CHO-K1 Cells Induced by 25-OHC Blocks by the Inhibitor of Arachidonate Metabolism, ETYA. CHO-K1 cells were seeded at 500 cells/35-mm culture dish in F12FC5 and incubated overnight. The culture medium was then changed to F12FC5 medium supplemented without or with 20 or 50 μ M of ETYA, and 0.03% EtOH (control) or 3 μ g/ml 25-OHC and incubated for 36 h. The medium was removed, and the cells were rinsed and re-fed F12FC5 for 5 days, and then surviving colonies were fixed, stained, and counted.

Arachidonate Metabolites Formed in Response to 25-OHC in CHO-K1 Cells

Activation of cPLA₂ results in the liberation of arachidonic acid. Arachidonic acid is a precursor for biosynthesis of eicosanoids, including prostaglandins, thromboxanes, leukotrienes, and lipoxins. It has been reported that 25-OHC treatment of arterial endothelial and smooth muscle cells produces a dramatic increase in the incorporation of labeled arachidonate into total prostaglandins (Wohlfeil and Campbell 1999) and this is due, at least in part, to upregulation of prostaglandin G/H synthase (COX). These observations fit nicely with our observations that 25-OHC upregulates cPLA₂ and suggest that a prostaglandin may be the arachidonate derived second messenger of apoptosis. In a preliminary experiment to test the possibility that a prostaglandin is the arachidonate product that signals apoptosis, we examined the effect of the cyclo-

oxygenase inhibitor indomethacin on 25-OHC cytotoxicity. As was the case with ETYA, indomethacin blocked 25-OHC cytotoxicity (Figure 15), consistent with the hypothesis that a prostaglandin could be in the signal trasduction pathway of oxysterol-induced apoptosis.

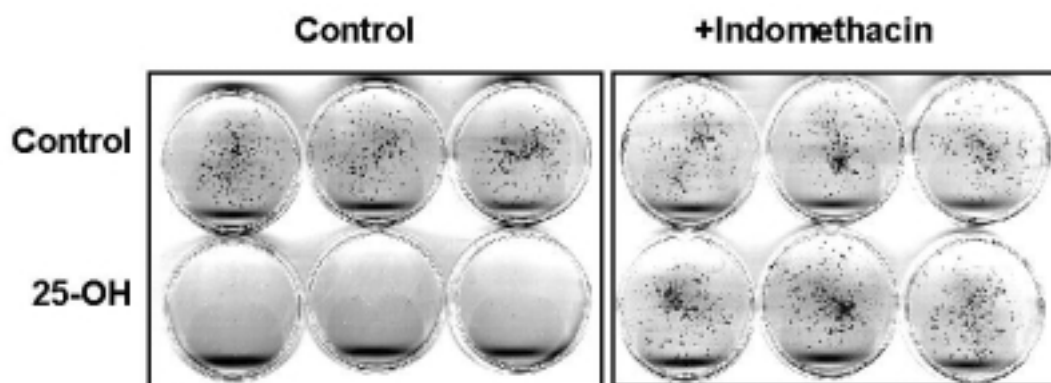


Figure 15. The Cyclo-Oxygenase Inhibitor, Indomethacin, Blocks the Cytotoxic Effects of 25-OHC. CHO-K1 cells were seeded at 500 cells/35-mm culture dish in F12FC5 and incubated overnight. The culture medium was then refreshed with medium supplemented with indomethacin (50 μ M), and 0.03% EOH (control) or 3 μ g/ml 25-OHC and incubated for an additional 36 h. The medium was then changed to standard culture medium and the cells were incubated for 5 days. The colonies derived from surviving cells were fixed, stained, and counted.

It has been found that prostaglandins of the J series in particular cause tumor cell apoptosis (Chinetti *et al.* 1998). To characterize the arachidonate metabolites formed in response to the treatment of 25-OHC, we labeled CHO-K1 cells with radioactive arachidonate and then examine the conversion of radioactive arachidonate to products in 25-OHC treated CHO-K1 cells by radio-HPLC. The result (Figure 16) shows that the major prostaglandin product, formed in response to treatment with 25-OHC, co-migrated with 15-deoxy- Δ 12,14 prostaglandin J₂ (15d-PGJ₂).

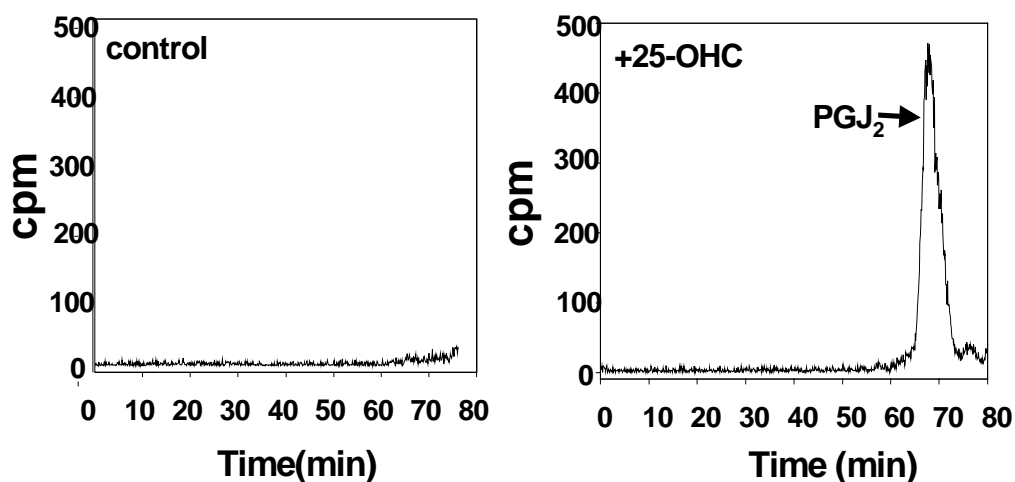


Figure 16. Effect of 25-OHC on the Metabolism of [^3H]Arachidonic Acid in CHO-K1 Cells. HPLC profile of radiolabeled eicosanoids resolved with HPLC method as described in Material and Methods. Main eicosanoid peak retention times are identified. A, Control; B, after treatment with 5 $\mu\text{g/mL}$ 25-OHC for 20 h

25-OHC and AA Metabolite 15-PGJ₂ Activate PPAR γ Mediated Cytotoxicity and Transcription

15d-PGJ₂ is a bioactive prostanoid produced by dehydration and isomerization of PGD₂, a cyclooxygenase product (Li *et al.*, 2001). It was recently shown to be an inducer of apoptosis in number of cell types including a variety of tumor lines (Keelan *et al.* 1999; Takahashi *et al.* 1999; Clay *et al.* 2001), vascular endothelial cells (Bishop-Bailey *et al.* 1999) and monocyte derived macrophage (Chinetti *et al.* 1998). The signal transduction pathway has been reported to be mediated through activation of peroxisome proliferator-activated receptor gamma (PPAR γ) (Chinetti *et al.* 1998). 15d-PGJ₂ is a naturally occurring ligand of PPAR γ (Forman *et al.* 1995; Kliewer *et al.* 1995). It has been reported, the activity of 15d-PGJ₂ as a PPAR γ ligand makes it cytotoxic (Chinetti *et al.* 1998). Because 15d-PGJ₂ showed significant cytotoxicity to CHO-K1 cell

as determined by clonegenic assay (Figure 17), we examined whether this apoptosis is through the activation of the PPAR γ pathway in CHO-K1 cells.

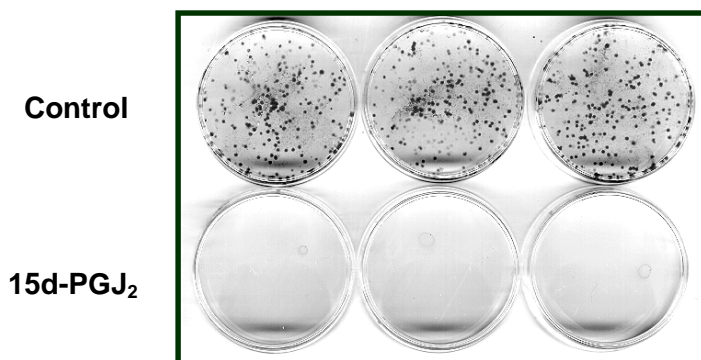


Figure 17. The Cytotoxicity of AA Metabolite 15d-PGJ₂. CHO-K1 cells were seeded at 500 cells per 35-mm culture dish in F12FC5 and incubated overnight. The culture medium was then changed to F12FC5 medium containing 0.05% DMSO (control) or 5 μ M 15d-PGJ₂ and incubated for 6 days before being fixed and stained.

To determine whether PPAR γ is active in CHO-K1, we transfected the CHO-K1 cells with the peroxisome proliferator response element (PPRE) luciferase reporter (AOX)3 TK-Luc, which contains three copies of the acyl-CoA oxidase PPRE cloned upstream of the TK-LUC reporter (a kind gift of Dr. C Glass, UCSD) and measured the luciferase activity. Treatment with 15d-PGJ₂ (5 μ M) or 25-OHC (5 μ g/mL) in these stably transfected cells with this reporter induced the PPRE-driven luciferase activity (Figure 18). These data strongly suggest 25-OHC and 15d-PGJ₂ activate PPAR γ -dependent transcriptional responses in CHO-K1 cells.

We next determined if activation of PPAR receptors by 25-OHC requires the activation of cPLA₂. We tested the effect of an inhibitor of arachidonate metabolism, ETYA. As shown in Figure 19, the activation of the PPAR γ response element reporter

gene in CHO-K1 cells by 25-OHC was blocked by ETYA. These data provide evidence that activation of PPAR γ by 25-OHC is the downstream of cPLA $_2$ activation.

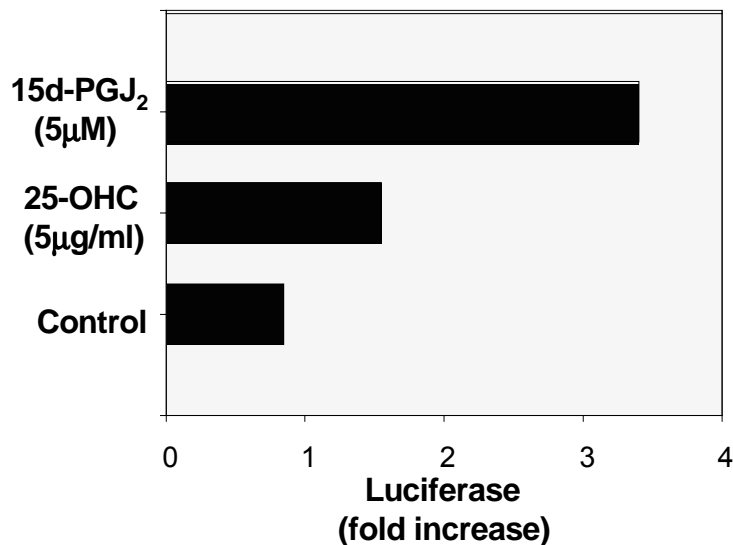


Figure 18. Activation of the PPAR γ Response Element Reporter Gene by 15d-PGJ $_2$ and 25-OHC in CHO-K1 Cells. The reporter construct contain three copies of the acyl-CoA oxidase PPRE cloned upstream of the TK-LUC reporter. Cells were cotransfected with PPRE x 3-TK-LUC and selected marker pWL-NEO. Stably transfected Cells were then treated with 5 μ M 15d-PGJ $_2$ and 5 μ g/ml 25-OHC for 24 h. Normalized luciferase activity were determined and plotted as fold activation relative to untreated cells.

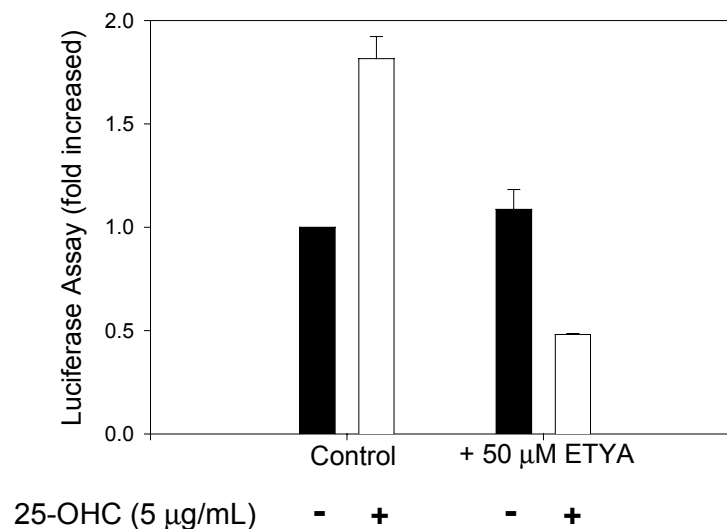


Figure 19. ETYA Blocks the Activation of PPAR γ Response Element Reporter Gene Stimulated by 25-OHC in CHO-K1 Cells. The cells stably transfected with PPRE x 3-TK-LUC as described in Materials and Methods were incubated for 24 h with F12FC5 medium supplemented with/without ETYA (50 μ M) and 25-OHC (5 μ g/mL) as indicated. Normalized luciferase activity were determined and plotted as fold activation relative to untreated cells.

25-OHC Treatment Results in Release of Cytochrome C from Mitochondria in CHO-K1 Cells

We further explored the execution phase of apoptosis induced by 25-OHC. The execution phase of apoptosis is comprised of those processes that commit cells to apoptotic death. Many independent studies have implicated mitochondria as a critical role in apoptotic execution (Newmeyer *et al.* 1994; Kroemer *et al.* 1997; Bossy-Wetzel *et al.* 1998). The activation of caspase-3 and subsequent late stage degradative events are probably triggered by the release of cytochrome C from the intermembrane space of mitochondria (Kroemer *et al.* 1997; Rosse *et al.* 1998; Susin *et al.* 1998).

PPAR γ has been demonstrated to induce apoptosis by interfering negatively with the anti-apoptotic NF κ B pathway (Beg and Baltimore 1996; Van Antwerp *et al.* 1996; Wang *et al.* 1996) through down regulation of Bcl-xL (Motoyama *et al.* 1995; Okada *et al.* 1998; Lee *et al.* 1999) that activates the cytochrome C release apoptotic pathway (Shimizu *et al.* 1999). The above results show that 25-OHC activates PPAR γ . The question is whether 25-OHC induces apoptosis by activating cytochrome C release. To answer this question, CHO-K1 cells were treated with 25-OHC and its effects on the release of cytochrome C from mitochondria into the cytosol were analyzed by Western Blots. The mitochondrial and cytosolic fractions were isolated and analyzed for levels of cytochrome C. The mitochondrial cytochrome C levels were decreased and cytosolic cytochrome C increased after CHO-K1 cells were treated 24 h with 25-OHC, demonstrating that cytochrome C had been released from mitochondria into the cytosol (Figure 20).

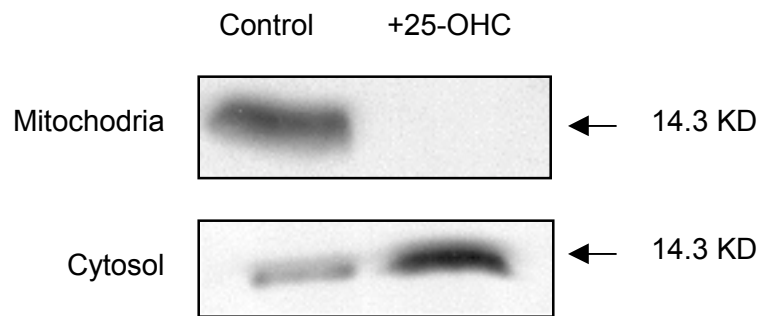


Figure 20. 25-OHC Induces Release of Cytochrome C from Mitochondria to Cytosol. Cells (2×10^6) treated with 0.03 % EtOH (control) or 3 μ g/ml 25-OHC for 24 h were harvested by centrifugation at 800 x g for 10 min at 4 °C. Mitochondrial and cytosolic extracts were prepared as described under Materials and Methods. The protein samples (50 μ g/lane) of mitochondria and cytosol were analyzed by immunoblotting, after SDS-PAGE (12 % polyacrylamide). Visualization was by ECL.

Caspase-3, -8, and -9 are Activated by Treatment of CHO-K1 Cells with 25-OHC

Cytochrome C can initiate the activation cascade of caspases once it is released into the cytosol (Budihardjo *et al.* 1999). Caspases can be grouped according to their substrate specificities, which are largely determined by the amino acids preceding the cleavage site aspartic acid residue. The substrate specificity of one group of caspases that includes caspases-8 and -9 is (V/L)EXD, a site similar to that found in caspase proenzymes (Thornberry *et al.* 1997). Therefore, these caspases may function as initiators of a proteolytic cascade by activating pro-caspases to amplify a death signal. In contrast, the substrate specificity of a second group of caspases, which includes caspase-3 is DEXD, a cleavage site that found in many target proteins that are cleaved during the effector phase of cell death (Talanian *et al.* 1997; Thornberry *et al.* 1997). During apoptosis, caspases are activated (Dixit 1996), and it has been suggested, based on inhibition of apoptosis by caspase-3 inhibitors, that during oxLDL (Harada-Shiba *et al.* 1998) or oxysterol (Harada *et al.* 1997; Harada-Shiba *et al.* 1998)-induced apoptosis, caspase-3 is activated in endothelial cells and macrophages. Therefore, we determined which caspase pathways are activated during induction of apoptosis in CHO-K1 cells.

Caspase-9 has been proposed to be the first caspase in the post-mitochondrial death pathway (Kuida 2000) and can directly activate the caspase-3 zymogen (Kuida 2000). If oxysterols activate mitochondrial route, caspase-9 should be activated. To confirm this, the enzymatic activity of caspase-9 in the cytoplasm of CHO-K1 cells was assayed with a fluorometrically labeled peptide substrate (Ac-LEHD-AFC). The results showed that 25-OHC treatment resulted in significant activation of caspase-9 (Figure 21). Activation of caspase-3 was also studied. The enzymatic activity of caspase-3 in the cytoplasm of CHO-K1 cells was assayed with a fluorometrically labeled peptide

substrate (Ac-DEVD-CMV). The results showed that 25-OHC treatments induced time-dependent activation of caspase-3 (Figure 22).

The release of cytochrome C from mitochondria can occur in the death receptor-independent, "mitochondrial-initiated," apoptotic pathway or through a death receptor and Bid-dependent apoptotic pathways (Budihardjo *et al.* 1999). Apoptotic pathways which are death receptor dependent proceed through receptor binding and auto-activation of procaspase-8. In CHO-K1 cells, the death receptor pathway has only been demonstrated to be operative with transfected death receptors such as Fas (Lee and Chau 2001) and PEA-15 (Ramos *et al.* 2000). However, recent reports (Slee *et al.* 1999; Tang *et al.* 2000) have demonstrated that activation of caspase-8 can also occur downstream of activation of caspase-9 and -3. Our results showed that caspase-8 was actually activated after CHO-K1 cells were treated with 25-OHC (Figure 21) in the absence of death receptors. This is probably downstream of caspase-3.

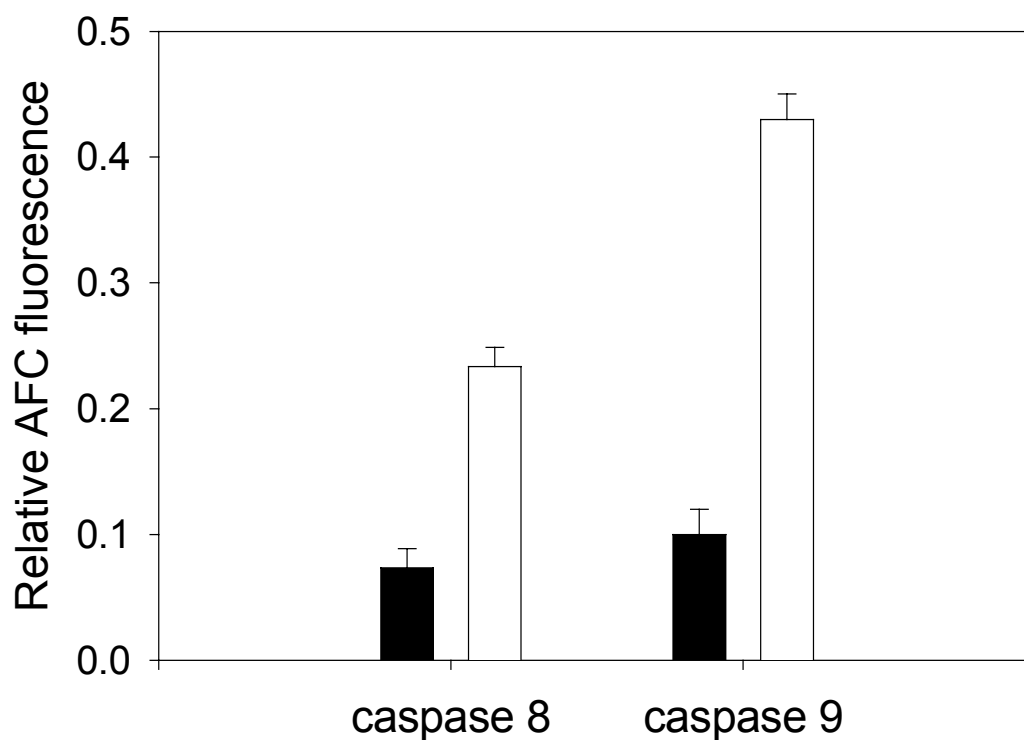


Figure 21. Activation of Caspase-8 and -9 by 25-OHC in CHO-K1 Cells. Cells (2×10^6) treated with 0.03% EtOH (control) or 3 $\mu\text{g/ml}$ 25-OHC for 24 h were harvested by centrifugation at 800 x g for 10 min at 4 °C. The cell pellets were washed once with ice cold PBS, lysed and 250 μg protein assayed for caspase-8 or caspase-9 activity for 1.5 h at 37 °C as described under Materials and Methods. The liberated AFC from Ac-IETD-AFC (substrate for caspase-8) or Ac-LEHD-AFC (substrate for caspase-9) was measured on a spectrofluorometer with an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

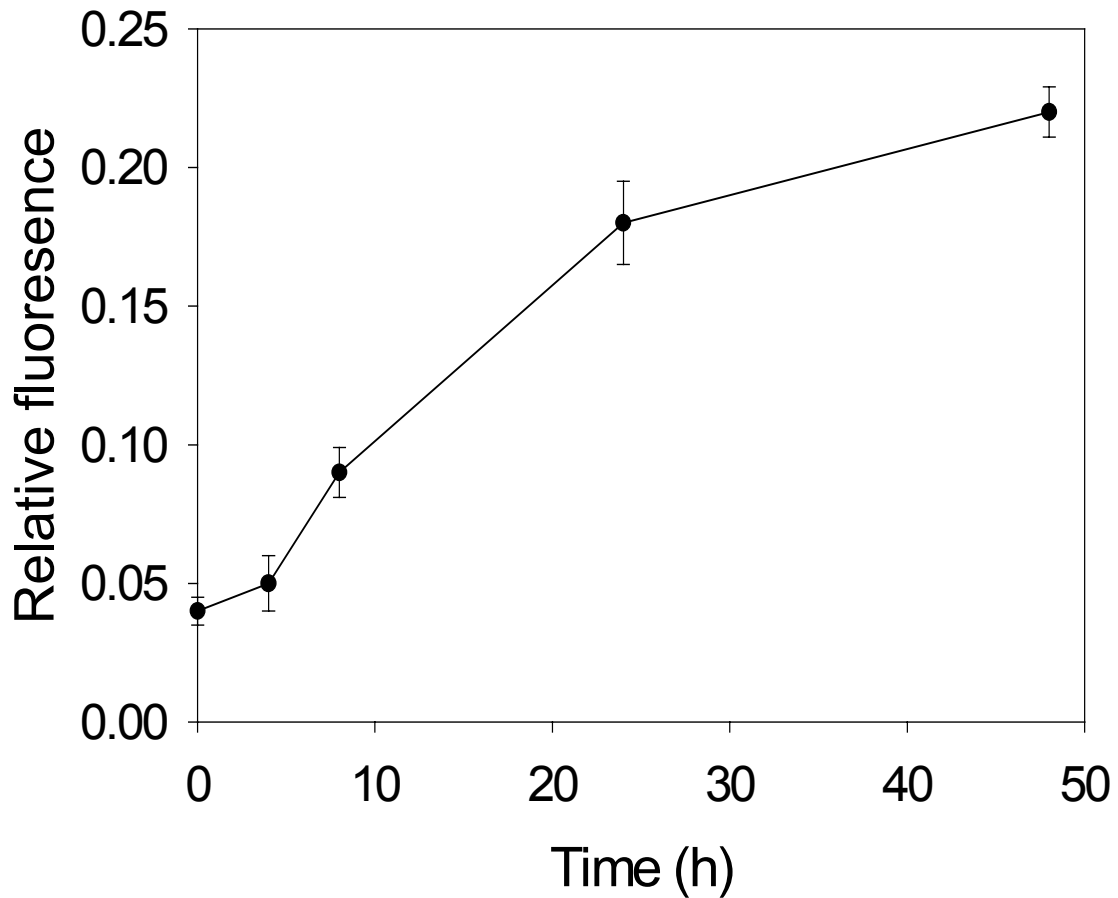


Figure 22. Time Course Caspase-3 Activity Induced by 25-OHC. Cells (2×10^6) treated with 0.03% EtOH (control) or 3 $\mu\text{g/ml}$ 25-OHC for various periods of time, as shown, were harvested by scraping with a rubber policeman followed by centrifugation at 800g for 10 min at 4 °C. The cell pellets were washed once with ice cold PBS and lysed with cell lysis buffer. 100 μg of total cell lysate protein were incubated with 20 μM , caspase 3 substrate, Ac-DEVD-AMC, 1.5 h at 37 °C in the protease assay buffer. The liberated AMC from Ac-DEVD-AMC was measured on a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 440 nm.

The Role of Caspase-3 in 25-OHC-Induced Apoptosis

Caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins in response to many apoptotic stimuli (Rudel and Bokoch 1997; Zhivotovsky *et al.* 1997; Levkau *et al.* 1998). To determine whether a caspase-3 substrate is actually being cleaved in CHO-K1 cells undergoing apoptosis, we examined the effect of 25-OHC treatment on poly (ADP-ribose) polymerase (PARP). PARP cleavage by caspase-3 is a common and physiologically significant step in the execution phase of apoptosis. The results (Figure 23) indicate that PARP cleavage is activated in CHO-K1 cells treated with 25-OHC. These results indicate that caspase-3 acts as a death mediator in 25-OHC-induced apoptosis.

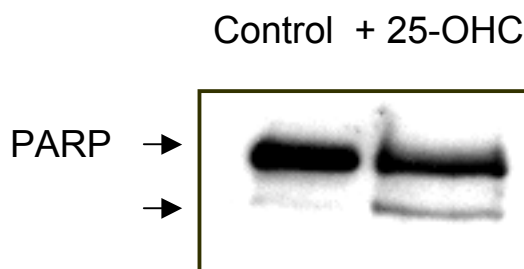


Figure 23. 25-OHC Induces Cleavage of PARP. Cells (2×10^6) treated with 0.03 % EtOH (control) or 3 $\mu\text{g/ml}$ 25-OHC for 24 h were harvested by scraping with a rubber policeman by centrifugation at $800 \times g$ for 10 min at 4°C . The cell pellets were washed once with ice cold PBS and nuclear extracts prepared as described under Materials and Methods. Nuclear protein (50 $\mu\text{g/lane}$) were resolved by 7.5 % SDS-PAGE and visualized by ECL immunoblotting.

The specific tetrapeptide inhibitor Ac-DEVD-CHO of caspase-3 was also used to further examine the role of caspase-3 in 25-OHC-induced apoptosis. As showed in Figure 24, Ac-DEVD-CHO significantly inhibited the generation of apoptotic cells in response to 25-OHC treatments, confirming that 25-OHC induces apoptosis by activating caspase-3 is an essential event in the apoptotic pathway, as is the case for oxLDL-induced apoptosis in vascular cells (Dimmeler *et al.* 1997).

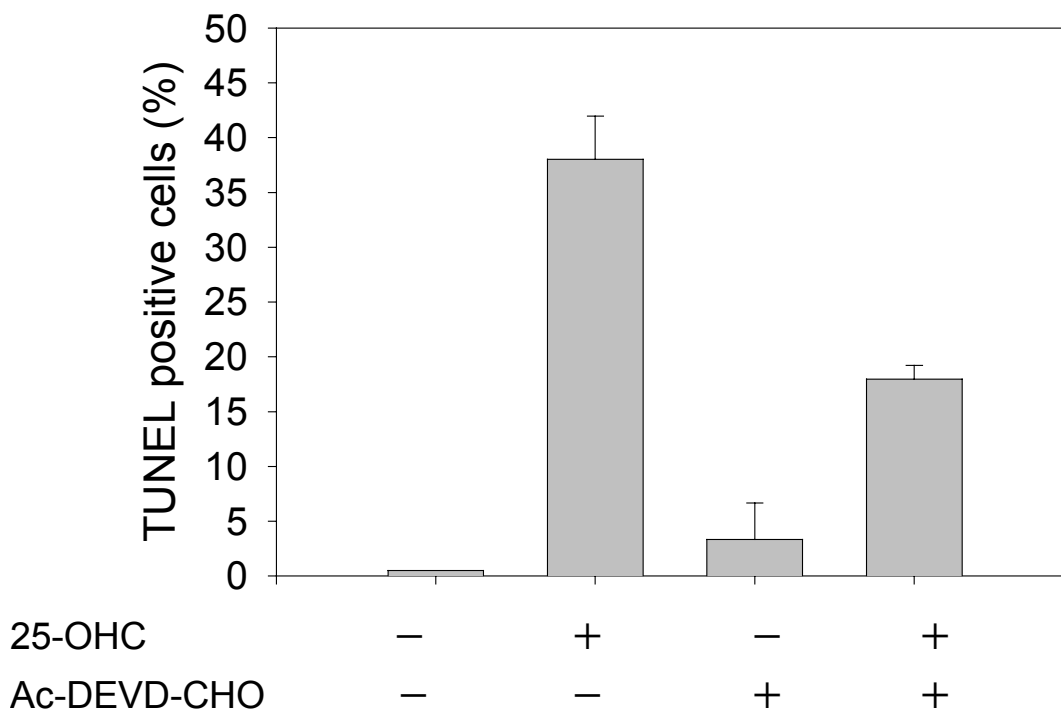


Figure 24. Inhibition of Caspase-3 Inhibitor Ac-DEVD-CHO on CHO-K1 Cell Apoptosis Induced by 25-OHC. Cells (50,000) were plated on glass coverslips and incubated for 24 h in F12FC5 containing 100 μ M caspase-3 inhibitor Ac-DEVD-CHO with 0.03% EtOH or 3 μ g/ml 25-OHC. After washing with PBS, the cells were fixed, and apoptosis was evaluated by an in situ TUNEL assay as described under "Materials and Methods". The graph represents the average percentage of TUNEL positive cells in 10 40x fields \pm S.D.

25-OHC can activate both cPLA₂ and caspase-3. The question is whether 25-OHC mediates caspase-3 activation through an activation of cPLA₂ process. The effect of blockage of 25-OHC stimulated arachidonate release on activation of caspase-3 was examined. We have demonstrated that the stimulated arachidonate release by 25-OHC is blocked by AACOCF₃ in CHO-K1 cells. We, therefore, examined the effect of 25-OHC treatment on caspase-3 activation in AACOCF₃ treated CHO-K1 cells. We found (Figure 25) that caspase-3 activity was inhibited after activation of cPLA₂ with 25-OHC blocked by AACOCF₃. This result indicates that activation of caspase-3 requires activation of cPLA₂

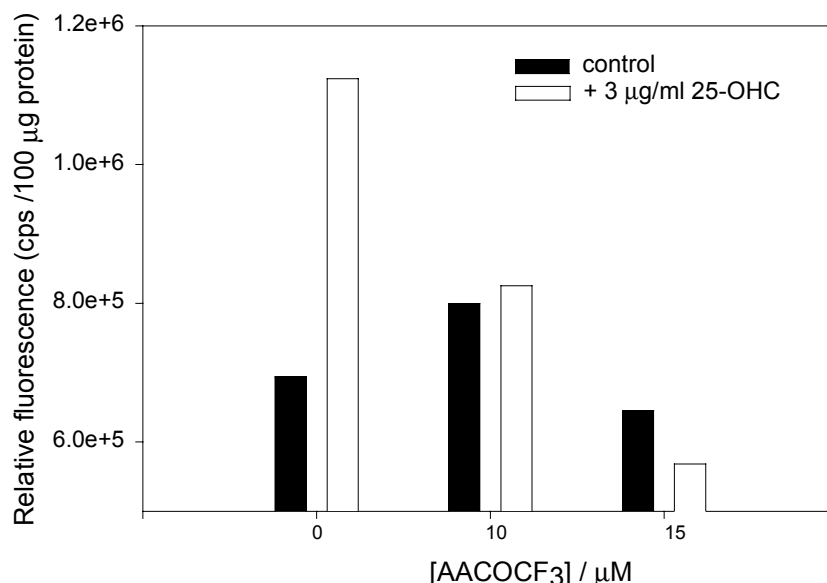


Figure 25. The cPLA₂ Inhibitor AACOCF₃ Blocks the Stimulation of Caspase-3 Activity by 25-OHC. CHO-K1 cells (2×10^6) were treated with 0.03% EtOH (control) or 3 μg/ml 25-OHC for 24 h in culture medium with or without various concentration of AACOCF₃ as shown. Cells were then harvested by scraping with a rubber policeman followed by centrifugation at 800 x g for 10 min at 4 °C. The cell pellets were washed once with ice cold PBS and lysed with cell lysis buffer. 100 μg of total cell lysate protein were incubated with 20 μM, caspase-3 substrate, Ac-DEVD-AMC, for 1.5 h at 37 °C in the protease assay buffer. The liberated AMC from Ac-DEVD-AMC was measured on a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 440 nm.

CHAPTER 4

DISCUSSION

The most obvious characteristic of atherosclerotic lesions is an abundance of cholesterol and other lipidic material that, in advanced lesions, is often referred to as "gruel". It is not fully understood, however, why this material persists rather than being cleared by the professional phagocytes such as monocytes/ macrophages that typically abound in such lesions. Recent studies have centered on the idea that components of these atherosclerotic lesions may be cytotoxic and, in particular, cause dysfunction and death of monocyte/ macrophage cells recruited to the site (Li *et al.* 2001). Large numbers of apoptotic monocytes/macrophages have been found in human atherosclerotic lesions (Mitchinson *et al.* 1996; Brown and Jessup 1999; Li *et al.* 2001). The progressive cell death within atheroma lesions has been suggested to result from oxLDL. OxLDL, which contains oxidized apoproteins as well as oxidized fatty acid and oxysterols, is present in substantial amounts in human atherosclerotic lesions. Several studies have shown that oxysterols and oxidized LDL induce apoptosis in a variety of vascular cell types. However, the precise mechanism involved in the oxLDL/oxysterols-induced apoptotic process is only poorly understood (Escargueil-Blanc *et al.* 1997).

In order to investigate the mechanism by which oxLDL induces apoptosis, we used a somatic cell genetic approach to determining the pathway of oxysterol induced apoptosis. Such an approach best proceeds through modeling complex regulatory phenomena in simple fibroblast models. Such fibroblast cell lines, particularly CHO-K1 cells (Leonard and Sinensky 1988), are readily amenable to both somatic cell and molecular genetic analysis. It is possible to use transfected or mutant CHO cell lines for genetic analysis of the gene products and functions involved in the programmed cell death induced by oxLDL or oxysterols. In order to rationalize the utilization of a

fibroblast (CHO-K1) model to study the regulation mechanisms involved in oxLDL apoptosis, we first explored the relationship between the vascular cell results on apoptosis and oxLDL induction of apoptosis in CHO-K1 cells.

To do this, we examined the hypothesis that oxLDL cytotoxicity exhibits specificity towards particular cell types (vascular smooth muscle cells, vascular endothelial cells, and macrophage) because of the presence of specific scavenger receptors on their surface. An extension of this notion is that cytotoxic oxysterols found in oxLDL can induce apoptosis in any cell type. If the data support these hypotheses, then utilization of the CHO-K1 cell for study of the oxysterol induction of apoptosis becomes credible.

We examined the cytotoxicity of oxLDL towards CHO-K1 cells bearing a receptor with high affinity for oxLDL, CD36. CD36 is a glycoprotein with a molecular mass of 88 kDa. CD36 is normally expressed in platelets, monocytes, macrophages, capillary endothelial cells, and adipocytes. The physiological function of CD36 has not been completely elucidated. Although there has been some controversy concerning the forms of LDL that bind to CD36, the ability of CD36 to bind oxLDL has been well documented (Endemann *et al.* 1993; Acton *et al.* 1994; Calvo *et al.* 1998). CHO-K1 cells were transfected with the CD36 expression vector to create the cell line CHO-K1/CD36. These cells readily take up oxLDL, in contrast to the parental cells which have no receptor that can recognize oxLDL (Figure 1). We found that the uptake of oxLDL in CHO-K1/CD36 was cytotoxic, in contrast to the parental cells, utilizing colony formation from single cells as an assay for cytotoxicity (Figure 2). As is the case for vascular cells bearing scavenger receptors for oxLDL, the pathway of cell death is apoptotic which has been demonstrated both by the TUNEL and DNA laddering assays (Figure 3). The single cell plating assay or colony formation from single cells for cytotoxicity and the *in situ* TUNEL assay for apoptosis used in this study are dependent on cell attachment. In

the former, live cells that detach before the end of the treatment will not form colonies and therefore will not be counted, producing artificially high estimations of cell death. In the apoptosis assay, on the other hand, detached cells will not be subjected to the TUNEL reaction, and artificially low estimation of apoptotic cell counts may be obtained.

The finding, that oxidized LDL induced apoptosis in a fibroblast cell line transfected with the human CD36 scavenger receptor, indicates that the apoptotic pathway, which mediates oxidized LDL cytotoxicity, is not tissue-specific. Tissue specificity is conferred by the presence of oxidized LDL receptors which mediate the uptake of oxidized LDL and, therefore, deliver large amounts of oxysterols to cells that possess them. We conclude that expression of CD36 in CHO cells is sufficient to confer on these cells the ability to take up oxLDL and to render them susceptible to killing by copper-oxidized LDL. Thus, the apoptotic pathway which mediates oxLDL-induced apoptosis is probably common to most cells.

We also showed here that oxysterols are cytotoxic to CHO-K1 cell. Several studies have examined the toxicity of oxysterols on various cell types of the vascular wall, including endothelial cells (Ramasamy *et al.* 1992), smooth muscle cells SMC (Peng *et al.* 1979; Hughes *et al.* 1994), and monocyte-macrophages (Aupeix *et al.* 1995; Clare *et al.* 1995). In these studies, a wide variety of effects and toxicity strength among the different oxysterols was observed (Lizard *et al.* 1999). We selected 25-hydroxycholesterol for our studies, because it is widely used as a model of this class of compounds. Again, we used colony formation from single cells as an assay for cytotoxicity (Figure 4) and *in situ* TUNEL and DNA laddering assays for apoptosis (Figure 5). The results clearly indicate that 25-hydroxycholesterol induces apoptosis.

Our overall hypothesis was that the induction of apoptosis by oxLDL is mediated by oxysterols. Because there is no direct evidence that oxysterols contribute to the cytotoxicity of oxLDL, a CHO-K1/CD36 mutant cell line resistant to killing by 25-

hydroxycholesterol (OX^{R}) was isolated. We, therefore, elected to determine whether this oxysterol resistant mutant is also cross-resistant to oxLDL. Indeed, we found that cells which were resistant to induction of apoptosis by 25-hydroxycholesterol were cross-resistant by oxLDL (Figure 6). This finding is, in our opinion, strong evidence supporting the notion that oxysterols are the component in oxLDL that induces apoptosis in CHO-K1 cells. In another words, oxysterols are at least partially responsible for the cytotoxic effects of oxidized LDL. It also suggests that the elucidation of the precise mechanism by which oxysterols can induce apoptosis would be of interest. Nevertheless, CHO-K1 fibroblasts appear to constitute a valuable model with which to explore the detailed mechanisms of oxLDL and oxysterol-induced cell death.

Apoptosis is generally triggered through complex sequences of intracellular signaling. The ability of 25-OHC to activate an apoptotic pathway in cells growing in medium supplemented with lipoproteins raises some additional questions regarding our understanding of the signaling pathways entrained by oxysterols. Oxysterols have only recently been recognized as signaling molecules. The best-described biological activity of oxysterols is the regulation of proteolytic maturation of the SREBPs and consequent transcriptional regulation of promoters bearing SREs. However, the apoptotic response to oxysterols does not appear to occur through their regulatory effects on SREBP processing and lipid metabolism. This notion is immediately suggested by the observation of apoptosis in the presence of exogenous lipoproteins but is also clearly supported by the differential responses of the cholesterol regulatory, CR1, and apoptosis-resistant OX^{R} , mutants to 25-hydroxycholesterol. Particularly noteworthy is the observation of normal oxysterol regulation of an SRE reporter expressed in OX^{R} , which strongly argues against any defect in the SREBP pathway in this type of resistant mutant.

Intracellular Ca^{2+} , rather than transcriptional control of SREs, appears to mediate the apoptotic effects of 25-hydroxycholesterol. The importance of calcium signals for initiating cell death has been demonstrated in many experimental systems (Juntti-Berggren *et al.* 1993), including in response to treatment of cells with 25-hydroxycholesterol (Ares *et al.* 1997; Ayala-Torres *et al.* 1997). In work reported by others, oxLDL-induced apoptosis has been demonstrated to be through a calcium-dependent pathway (Escargueil-Blance *et al.* 1997). Consistent with the proposition that oxysterols mediate for most of oxLDL apoptotic activity, it appears that calcium influx through plasma membrane channels is an important signal in oxysterol-induced apoptosis (Ares *et al.* 1997). Our results on CHO-K1 cells confirmed this observation and showed that 25-hydroxycholesterol -induced apoptosis was apparently calcium-dependent as shown by inhibition of apoptosis when calcium-free medium was used for the treatments. Within a few minutes after addition, 25-hydroxycholesterol induced a 3-fold increase in intracellular calcium. However, a 25-hydroxycholesterol-resistant mutant, OX^{R} , did not exhibit enhanced Ca^{2+} uptake when treated with 25-hydroxycholesterol (Figure 9). These findings strongly suggest that increased intracellular calcium is a critical mediator of oxysterol toxicity.

The mechanisms underlying induction of apoptosis through Ca^{2+} mobilization remain partly elusive; at present there are 2 models to explain how alterations in Ca^{2+} homeostasis might trigger apoptosis (Kass and Orrenius 1999). In one, depletion of intracellular stores and possibly influx of Ca^{2+} across the plasma membrane promote a sustained Ca^{2+} increase that acts as a signal for apoptosis. In the second, it is not the Ca^{2+} increase but the emptying of intracellular Ca^{2+} stores that triggers apoptosis, perhaps by disrupting intracellular architecture and allowing key elements of the effector machinery to gain access to their substrates. Our data showed that calcium influx triggered by 25-hydroxycholesterol could be inhibited by calcium channel blocker

nifedipine (Figure 9B). Furthermore, we show that nifedipine diminished apoptosis induced by 25-hydroxycholesterol (Figure 9A). This observation of activation of Ca^{2+} uptake, through a nifedipine-inhibitable channel in CHO-K1 cells, is novel. The existence of a voltage-dependent, nifedipine-inhibitable calcium channel in CHO-K1 cells has previously been demonstrated (Skryma *et al.* 1994) by the patch-clamp technique. However, no functional role for this channel or regulator of its activity has previously been described.

The signal transduction pathway, distal to sustained increases in intracellular Ca^{2+} , proceeds through the activation of the 85-kDa cPLA₂. Activated cPLA₂ preferentially hydrolyzes phospholipids containing arachidonate at the sn-2 position, thus providing free arachidonic acid for the biosynthesis of biologically active eicosanoid products (Clark *et al.* 1991; Kramer *et al.* 1991). cPLA₂ activity is regulated both transcriptionally and post-translationally. Post-translational activation is thought to occur by two mechanisms. One mechanism involves a Ca^{2+} -dependent translocation of cPLA₂ from the soluble to the membrane fraction of cells, allowing cPLA₂ access to its arachidonyl-containing phospholipid substrate. Calcium plays a role by promoting binding of cPLA₂ to membrane, which is mediated by a calcium-phospholipid-binding domain (C2 domain) at the amino terminus of cPLA₂. In response to calcium increase, activation of cPLA₂ translocates from cytosol to the nuclear envelope and endoplasmic reticulum (Gijon and Leslie 1999). The second involves agonist-induced MAP kinase phosphorylation of cPLA₂, resulting in stimulation of its intrinsic enzymatic activity (Muthalif *et al.* 1996).

We have examined these general concepts of Ca^{2+} -dependent cPLA₂-mediated apoptosis and have found results consistent with the induction of apoptosis by 25-OHC through the activation of cPLA₂. 25-OHC could be shown to stimulate arachidonate release in CHO-K1 cells (Figure 10). The susceptibility of the release to inhibition by

AACOCF₃ but not by BEL is consistent with the release occurring through activation of cPLA₂. The data presented here establish that in intact cells, cPLA₂ translocates from cytosol to membranes in response to the treatment of 25-hydroxycholesterol (Figure 12). Specifically, cPLA₂ binds to the nuclear envelope and the endoplasmic reticulum. No evidence of activation of cPLA₂ by phosphorylation was observed (Panini *et al.* 2001).

That the arachidonate release is calcium dependent could be demonstrated in CHO-K1, where we demonstrated a requirement for calcium influx in the induction of apoptosis by 25-OHC. This conclusion is supported by the loss of arachidonate release in response to 25-OHC after treatment with nifedipine (Figure 11), which we have shown blocks calcium influx and apoptosis in response to 25-OHC treatment in CHO-K1 cells (Figure 9A and B). This conclusion is also supported by the loss of stimulation of arachidonate release in 25-OHC-resistant mutant OX^R cells (Figure 11), which do not increase calcium uptake in response to 25-OHC treatment (Figure 9C). The calcium dependence of the stimulation of arachidonate release is also characteristic of cPLA₂. Furthermore, blockage of arachidonate release by AACOCF₃ eliminated the cytotoxic effects of 25-OHC in CHO-K1 cells (Figure 13). The observations are consistent with 25-OHC activating cPLA₂ and also suggest that the activation of this enzyme is in the apoptotic signal transduction pathway triggered by these agents.

PLA₂ enzymes have been proposed to play a role in mediating apoptosis in various models, although the specific PLA₂ enzyme involved appears to depend on the cell model and the agents used to induce cell death (Cummings *et al.* 2000). CPLA₂ has been shown to be involved in the cell death induced by TNF in cells sensitized to killing with either adenovirus infection or inhibitors of transcription/translation (Thorne *et al.* 1996; Voelkel-Johnson *et al.* 1996). CPLA₂ has also been shown to be crucial for TNF-induced cytotoxicity in a TNF-sensitive cell line, L929, but not to be involved in Fas-

mediated apoptosis (Hayakawa *et al.* 1993; Enari *et al.* 1996). It has been shown that cPLA₂ is cleaved and inactivated by caspases in HeLa cells undergoing apoptosis (Adam-Klages *et al.* 1998) and during Fas-mediated cell death in U937 cells (Atsumi *et al.* 1998). CPLA₂ has cleavage sites for caspase 1 and 3 and proteolysis at these sites disrupts the catalytic diad and inactivates the enzyme (Atsumi *et al.* 1998; Luschen *et al.* 1998). Rather than cPLA₂, which is inactivated, a role for the group VI iPLA₂ in Fas-mediated apoptosis has been proposed on the basis of the observation that iPLA₂ inhibitors suppress induction of arachidonic release and apoptosis by Fas. iPLA₂ also contains a caspase cleavage site but this cleavage enhances its catalytic activity (Atsumi *et al.* 2000). The enhanced iPLA₂ activity is proposed to accelerate turnover of phospholipids that may influence membrane changes that occur during apoptosis.

Our results clearly demonstrate that oxysterol-induced apoptosis is calcium-dependent process that involves activation of cPLA₂. In work reported by our laboratory (Panini *et al.* 2001), 25-hydroxycholesterol does not appear to induce a degradation of cPLA₂ in CHO cells, unlike the finding in certain other model of apoptosis (Atsumi *et al.* 1998; Luschen *et al.* 1998). It is notable that even after 24 h of 25-hydroxycholesterol exposure, a period in which we have shown 25-hydroxycholesterol activation of caspase 3 to be maximal, there is no loss of signal intensity of intact cPLA₂ bands in immunoblots.

Arachidonate can be oxidatively metabolized to diverse biologically active products. Regulation of AA metabolism is critical to the growth and survival of all cell types. ETYA blocks all of the known oxidative metabolic pathways including cyclooxygenase and all lipoxygenases (Tobias and Hamilton 1979; Bokoch and Reed 1981; Salari *et al.* 1984) as well as cytochrome P-450-dependent arachidonate metabolism (Capdevila *et al.* 1988). Rescue of CHO-K1 cells from killing by 25-OHC by ETYA (Figure14) is, therefore, consistent with a metabolite of arachidonate being a second

message of oxysterol-induced apoptosis but does not indicate which arachidonate oxidative pathway is involved. It has been reported that 25-OHC treatment of arterial endothelial and smooth muscle cells produces a dramatic conversion of labeled arachidonate into total prostaglandins (Wohlfeil and Campbell 1999) and this is due, at least in part, to upregulation of prostaglandin G/H synthase 2 (COX-2) (Wohlfeil and Campbell 1997, 1999). These reports fit with our finding that 25-OHC upregulates cPLA₂ and suggest that a prostaglandin may be the arachidonate-derived second messenger of apoptosis. Our observations of the inhibition of 25-OHC cytotoxicity by the cyclo-oxygenase inhibitor indomethacin is also consistent with this hypothesis (Figure 15).

Among COX-2-derived compounds, prostaglandins of the J series, and more specifically 15d-PGJ₂ is recognized as a potent apoptotic and growth inhibitory factor (Kliewer and Willson, 1998). Several studies have shown that 15d-PGJ₂ induces apoptosis in a variety of cell types (Chinetti *et al.* 1998; Bishop-Bailey and Hla 1999; Clay *et al.* 2001; Li *et al.* 2001). In this study, we have confirmed these observations and shown that 15d-PGJ₂ induced significant cytotoxicity to CHO-K1 cells as determined by clonegenic assay (Figure 17).

Apoptotic signaling by 15d-PGJ₂ has been reported to proceed through the activation of PPAR γ (Bishop-Bailey and Hla 1999). 15d-PGJ₂ is a metabolite of PGD₂ produced by dehydration of PGD₂. In contrast to classical prostaglandins, which bind to cell surface G protein-coupled receptors, 15d-PGJ₂ is a natural ligand for the nuclear receptor PPAR γ , which is currently considered a major receptor for these prostaglandins (Kliewer and Willson 1998; Spiegelman 1998). PPAR γ functions as a ligand-dependent transcription factor, which, upon heterodimerization with the 9-cis-retinoic acid receptor, binds to a specific response element, PPRE, thus regulating the expression of target genes (Schoonjans *et al.* 1997). Our results have shown that 15d-PGJ₂ induced the

activation of PPAR γ in CHO-K1 cells by demonstrating an increase in levels of PPRE-mediated transcription (Figure 18). These data confirm the fact that 15d-PGJ₂ is a potent activator of PPRE mediated transcription in CHO cells and that these cells express PPAR γ .

The finding that 25-OHC upregulates cPLA₂ and prostaglandins can serve as ligands for PPAR γ raised the possibility that 25-OHC could indirectly induce peroxisome proliferation by affecting prostaglandin metabolism to generate a PPAR γ ligand that could be involved in the transcriptional activation of genes. 25-OHC is known to regulate prostaglandin production in artery smooth muscle and endothelial cells (Wohlfeil and Campbell 1997, 1999). Thus, it appeared possible that in our model system, 25-OHC might regulate prostaglandin metabolism, resulting in the increased production of PPAR γ ligand. In fact, we found that 25-OHC administration enhanced arachidonic acid metabolism in the CHO-K1 cells, as indicated by conversion of labeled arachidonic acid to polar metabolites. One of metabolites behaved chromatographically similar to 15d-PGJ₂ in HPLC analyses (Figure 16). Furthermore, our transfection experiments demonstrated that 25-OHC showed an activation of PPAR γ similar to that by 15d-PGJ₂ in CHO-K1 cells as indicated by increasing levels of PPRE-mediated transcription, with a more than 2-fold increase in luciferase reporter activity of PPRE (Figure 18).

Activation of PPAR γ has effects on both metabolic risk factors and on vascular inflammation related to atherosclerosis. PPAR γ has profound effects on the differentiation and function of adipose tissue, where it is highly expressed (Tontonoz *et al.* 1994). PPAR γ is also expressed in atherosclerotic lesions (Neve *et al.* 2000; Plutzky 2000; Itoh and Nakao 2001). PPAR γ is present in vascular endothelial cells, smooth muscle cells, monocytes, and monocyte-derived macrophages (Chinetti *et al.* 1998; Neve *et al.* 2000). It may modulate the pathogenesis of atherosclerosis by regulating the expression of activated genes which are involved in vascular cell apoptosis. It has

been reported that ligands for PPAR γ were generated from oxLDL through scavenger receptor-mediated endocytosis followed by intracellular processing (Nagy *et al.* 1998). The endocytosis - and ligand-dependent activation of PPAR γ by oxLDL is responsible for regulating another transcriptional nuclear NF- κ B, which is involved in cell proliferation (Han *et al.* 2000). It is noteworthy that the ligand-dependent activation of PPAR γ antagonizes the activation of NF- κ B (Han *et al.* 2000; Nishio *et al.* 2000). This implies that the endocytic processing of oxLDL may function as an inactivation route for oxLDL-induced NF- κ B signaling through activation of PPAR γ . In work reported by others (Han *et al.* 2000; Li *et al.* 2001), PPAR γ activation results in a pronounced induction of apoptosis in human macrophages. Consistent with the proposition that oxysterols mediate most of oxLDL apoptotic activity, it appears that PPAR γ is an important signal in oxysterol-induced apoptosis. This conclusion is supported by the inhibition of PPAR γ activation in response to 25-OHC after treatment with the arachidonate metabolic inhibitor ETYA (Figure 19), which we have shown to block apoptosis in response to 25-OHC treatment in CHO-K1 cells (Figure 14). These observations strongly suggest that PPAR γ activation play an important role in the control of 25-OHC-induced apoptosis in CHO.

As reported in the literature, PPAR γ inhibition of NF- κ B can demonstrably induce apoptosis in lymphocytes through down regulation of a Bcl family member, Bcl-xL, activating the canonical cytochrome C release apoptosis pathway (Lee *et al.* 1999). Cytochrome C is synthesized in cells from a cytoplasmic mRNA and specifically imported into the mitochondria where it resides in the space between the inner and outer mitochondrial membranes. During apoptosis of many cell types, cytochrome C has been shown to be released from the mitochondrial inter-membrane space into the cytosol, an event that leads to caspase activation (Budihardjo *et al.* 1999; Chang and

Yang 2000). Our data demonstrated that 25-OHC treatment resulted in the release of cytochrome C from mitochondria into the cytosol in CHO cells (Figure 20).

The post-mitochondrial pathway is well documented. Cytochrome C together with ATP facilitates oligomerization and activation of Apaf-1 and caspase-9, leading to activation of caspase-3 and other effector caspases, which then cleave their substrates, finally leading to the apoptotic cell death (Zou *et al.* 1999). Consistent with the activation of such a pathway, caspase-9 enzymatic activity was increased in response to the treatment with 25-OHC (Figure 21). These findings suggest that 25-OHC activates the mitochondrial route.

Caspase-3, a member of the interleukin-1 β -converting enzyme-like protease family, has been identified as one of the key enzymes in apoptotic pathway (Salvesen and Dixit 1997). We found that caspase-3 enzymatic activity was increased up to 10-fold after 24 h treatment with 25-OHC (Figure 22). Activated caspase-3 has been proposed as a pivotal molecule in apoptosis because of its capacity to cleave protein substrates essential for cell survival. The most extensively studied substrate for caspase-3 is PARP, a key enzyme involved in genome surveillance and DNA repair. After caspase activation, PARP loses its ability to bind damaged DNA because of cleavage between two zinc-finger DNA binding motif (Decker *et al.* 2000). Loss of normal PARP has also been proposed to up-regulate Ca²⁺ and Mg²⁺-dependent endonuclease activity, which participates in the internucleosomal DNA cleavage in apoptosis (Yakovlev *et al.* 2000). Our results show that the intact 116-kDa PARP was processed to form the expected caspase-3 derived 85-kDa residual fragment after the cells were treated with 25-hydroxycholesterol for 24 h (Figure 23). Furthermore, addition of Ac-DEVD-CHO, a specific peptide aldehyde inhibitor of caspase-3, inhibited 25-OHC-induced apoptosis (Figure 24). These results suggest that the activation of caspase 3 is in the apoptotic signal transduction pathway triggered by 25-hydroxycholesterol

That the activation of caspase 3 is via an activation of cPLA₂ process could be demonstrated in CHO-K1, where we have demonstrated a requirement for arachidonate release in the induction of apoptosis by 25-OHC. This conclusion is supported by the inhibition of caspase-3 activity in response to 25-OHC after treatment with AACOCF₃ (Figure 25), which we have shown blocks arachidonate release and apoptosis in response to 25-OHC treatment in CHO-K1 cells (Figure 10 and 13). Taken together, these data provide strong evidence indicating that 25-OHC-induces caspase-3-mediated apoptosis via an activation of calcium-dependent cPLA₂. These findings also demonstrate that oxysterol induces apoptosis of CHO-K1 cells via the common execution phase-cytochrome C release from mitochondria into the cytosol and activation of caspases, which is in agreement with the previous reports that oxysterols and oxLDL could activate caspase-3 in human vascular cells.

From current understanding, there exist 2 well-characterized pathways transducing a death signal to the apoptotic machinery (Budihardjo *et al.* 1999). One is at the cell surface where death receptors transmit a signal across the cell membrane following receptor clustering. The second is the death receptor-independent apoptotic pathway, which is directly activated by a death signal and follows the release of cytochrome C from mitochondria. To distinguish which pathway is activated, the first recognizable biochemical event is specific caspase activation, and each uses distinct caspases. In the death receptor pathway(s), procaspase-8 (FLICE) is recruited to the cytosolic face of the receptor after ligand binds to the receptor (Muzio *et al.* 1996). The intrinsic proteolytic activity of procaspase-8 is sufficient to produce auto-proteolytic maturation when these molecules are brought into close proximity by receptor association (Martin *et al.* 1998; Muzio *et al.* 1998). Activated caspase-8 either can directly cleave and activate the effector caspase, or it can cleave Bid to induce the release of mitochondrial cytochrome C, which can activate effector caspases by

complex formation with Apaf-1 and caspase-9 in the presence of ATP (Li *et al.* 1998; Luo *et al.* 1998; Budihardjo *et al.* 1999; Gross *et al.* 1999). Because no prior literature indicates that CHO-K1 cells express death receptors, it would seem unlikely that the death receptor pathway could be activated by 25-OHC, although we cannot at this point formally rule out this possibility, particularly in light of our observation of activation of caspase-8. Activation of a death receptor pathway by 25-OHC could thus begin with activation of caspase-8 and go through Bid signal transduction to cytochrome C release and subsequent downstream caspase activation. However, it has also been reported (Slee *et al.* 1999) that, in vitro, caspase-8 activation can also take place downstream of caspase 9 and this seems to be part of the terminal execution phase of apoptosis (Tang *et al.* 2000). In view of the lack of known death receptors in CHO-K1 cells, we favor the possibility that activation of caspase-8 occurs downstream of caspase-3.

Based on our results and the results of others, we propose the following model for the induction of apoptosis by 25-OHC in CHO-K1 cells. Upon treatment of CHO-K1 cells with 25-OHC, 25-OHC induces calcium influx through an L-type calcium channel. A sustained increase in calcium can activate cPLA₂ and promote arachidonic acid release. cPLA₂-mediated liberation of AA leads to the formation of a cyclooxygenase product, prostaglandin, which can activate the transcription factor PPAR γ . According to the literature report, PPAR γ activation by 25-OHC is endocytosis-dependent, and that the ligand-dependent activation of PPAR γ antagonizes 25-OHC-induced NF- κ B activation. PPAR γ inhibition of NF- κ B can down-regulate Bcl-xL, which can activate the cytochrome C release apoptotic pathway. Our results show that 25-OHC induces the release of mitochondrial cytochrome C. Cytosolic cytochrome C facilitates the activation of caspase 9, 8, and 3. 25-OHC treatment induces the degradation of PARP- a substrate for caspase 3 and a key enzyme involved in genome surveillance and DNA repair.

All of the results are summarized in Figure 26. These results support the notion that our simple model system, treatment of CHO-K1 with 25-OHC, could be used to investigate the signaling pathway of vascular cell apoptosis by oxLDL that is thought to initiate atherosclerosis lesion development. These findings on the oxysterol/oxLDL-mediated signaling pathway in CHO cells may contribute to our understanding of atherogenesis and provide possible therapeutic interventions.

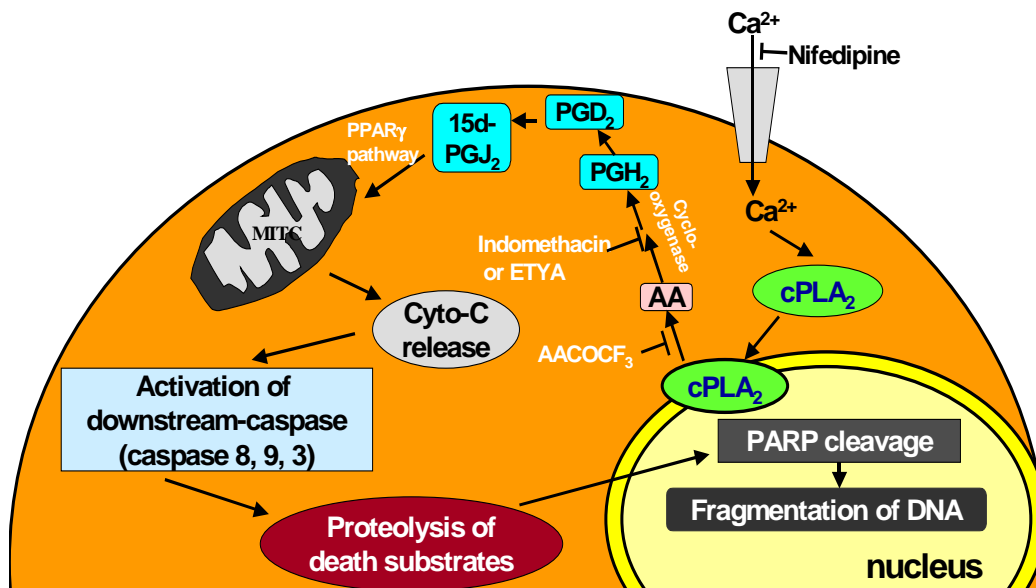


Figure 26. Summary of Signaling Pathway of Apoptosis Induced by 25-Hydroxycholesterol in CHO-K1 Cells.

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Honors and
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Outstanding Academic Achievement, East Tennessee State University, 1996, 1997, 1998, 1999, and 2000

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